REMARKS

Claims 1-3, 5-11, and 15-23 are pending after entry of this paper. Claims 1-4, 14-24, and 24 have been rejected. Claims 5-11 and 15-23 have been withdrawn and claims 4, 12-14, and 24 have been cancelled without prejudice. Applicants reserve the right to pursue withdrawn and cancelled claims in a divisional or continuing application.

Claims 1 and 2 have been amended. Specifically, claim 1 has been amended to incorporate the subject matter of the presently cancelled claim 4 and partially incorporate the subject matter of claim 2. Support may be found throughout the instant specification, for example, claims 2 and 4 as originally filed. Claim 2 has been amended to properly depend from the presently amended claim 1 with respect to the antibiotic rifampicin. Support may be found throughout the instant specification, for example, at page 16, lines 5-6.

No new matter has been introduced by these amendments. Reconsideration and withdrawal of the pending rejections in view of the above claim amendments and below remarks are respectfully requested.

Response to Rejections under 35 U.S.C. §112

Claims 1-4, 12-14, and 24 stand rejected under 35 U.S.C. §112, first paragraph for lack of enablement. Specifically, the Examiner contends that while the specification is enabled for the treatment of angiogenesis of retinal microvascular endothelial cells, the specification is allegedly not enabled for the treatment of other tumor types by administering rifampicin. Applicants respectfully disagree.

As an initial matter, applicants respectfully wish to remind the Examiner that the present invention as recited in claim 1 is directed to the inhibition of the angiogenesis by rifampicin in malignant tumors. Applicants assert that the question of enablement must address whether the instant application enables one skilled in the art to inhibit angiogenesis in malignant tumors and not, as argued by the Examiner, to treat and potentially cure cancers. Hence, the Examiner's argument that "the skilled artisan would have to accept that by administering the presently claimed compound rifampicin, all cancers … known in the art could be treated" (Office Action – page 4) is not directly related to the enablement of the present invention. As the Examiner aware, the angiogenesis is a process in which new blood vessels are generated from existing blood vessels, which may arise in the course of the tumor growth or in the process of wound heeling (*Nature Medicine* 1995:1;27-31). In the course of the solid tumor growth, the tumor needs the continues supply of nutrition and oxygen, which is accommodated by angiogenesis. Therefore, while the inhibition of angiogenesis is expected to be beneficial in treatment of solid tumors by cutting off the tumor's supply of nutrition and oxygen, the angiogenesis-inhibiting substances do not directly attack the tumor per se, contrary to the Examiner's contention. Nonetheless, applicants assert that rifampicin proved to be very effective in treating solid tumors by inhibiting angiogenesis in malignant tumors. Further to Example 6 mentioned by the Examiner (Office Action – page 3), applicants wish to direct the Examiner's attention to Examples 4 and 5. Example 4 shows that growth of the tumor derived from colon cancer is inhibited by rifampicin, and Example 5 shows that episodes of liver cancer are inhibited by rifampicin. Furthermore, in the subsequent studies, applicants determined that rifampicin has an advantageous effect on Lewis lung cancer and metastasis thereof, and an advantageous effect on metastasis of A549 lung cancer to the liver (Japanese Patent Application No. 2007-034960;

can be supplied per Examiner's request). Finally, as noted in the specification at pages 5 and Figure 1, rifampicin shows endostatin-type angiogenesis-inhibiting signals. In the review article, Folkman teaches that endostatin is effective in inhibiting angiogenesis and the treatment of significantly various types (65 different types) of cancers. (*Experimental Cell Research*, 312 (2006) 594 - 607, attached as Exhibit 1). Therefore, contrary to the Examiner's contention, the effectiveness of rifampicin in the inhibition of angiogenesis of malignant tumors is fully enabled by the instant disclosure and the knowledge in the art without necessitating an undue level of experimentation suggested by the Examiner (Office Action – page 8) Therefore, the applicant asserts that the claimed invention is fully enabled for the entire scope of the presently claimed subject matter, *i.e.*, inhibition of angiogenesis in malignant tumors. Reconsideration and withdrawal of the rejection under 35 U.S.C. §112, first paragraph are respectfully requested.

Response to Rejections under 35 U.S.C. §102

Claims 1-4, 12-14, and 24 have been rejected under 35 U.S.C. §102(b) as being anticipated by Demkow et al. (*Pneumonologia I Alergologia Polska* 1998). Specifically, the Examiner contends that Demkow teaches that rifampicin allegedly inhibits angiogenesis. (Office Action – page 9). Applicants respectfully disagree.

Applicants respectfully wish to remind the Examiner that "[a] claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." (see MPEP 2131). Contrary to the Examiner's contention, Demkow does not teach each and every element of the claimed invention as presented in the independent claim 1. In particular, claim 1 recites that the inhibition of

angiogenesis occurs in malignant tumors. Demkow, on the other hand, teaches the effects of rifampicin on angiogenesis in mononuclear leukocytes (see Table III of Demkow). Applicants respectfully assert that the angiogenesis in leukocytes (*i.e.*, leukocyte-induced angiogenesis or LIA) and the angiogenesis in malignant tumors (*i.e.*, tumor induced angiogenesis or TIA) are completely different and are not interchangeable. A skilled artisan would not and could not look to Demkow for the teaching of inhibiting the angiogenesis of malignant tumors with rifampicin based on the teaching of inhibiting the angiogenesis of leukocytes.

It was well recognized in the art that angiogenesis normally arise in tumors or in the process of wound heeling (*Nature Medicine* 1995:1;27-31). However, angiogenesis may also be affected and caused by an inflammation. For instance, when polymorphonuclear leukocyte activated by N-formyl-methionyl-leucyl-phenylalanine adheres to vascular endothelium, angiogenesis effect is enhanced through adhesion molecules, such as ICAM-1 or E-selectin (Yasuda et al., *Life Sci* 2000:66;2113-2121, attached as Exhibit 2). According to Yasuda, the ICAM-1 and E-selectin are involved in the induction of angiogenesis in leukocytes because anti-ICAM-1 and anti-E-selectin antibodies inhibit leukocyte-induced angiogenesis, which readily demonstrates a relationship between inflammation and leukocyte-induced angiogenesis (*Am J Physiol* 2002:282;C917-C925, attached as Exhibit 3). On the other hand, since angiogenesis can arise without inflammation, the mechanism of angiogenesis in malignant tumor has been thought to differ from that of leukocyte-induced angiogenesis. In fact, Malkowska-Zwierz (also co-authored by Demkow; cited by the Examiner) suggests that rifampicin does not inhibit angiogenic activity of tumor cells but only lucocyte-induced angegonesis. (Malkowska-Zwierz et al., *International Journal Of Oncology*, vol. 7, 1995, page 968; attached as Exhibit 3).

Therefore, applicants assert that a skilled artisan would not have the knowledge to conclude based on the effect of rifampicin in inhibiting leukocyte-induced angiogenesis, it could also inhibit tumor induced angiogenesis. In fact, the subsequent publication co-authored by Demkow teaches away from the claimed invention by explicitly teaching that rifampicin did not inhibit tumor induced angiogenesis. Therefore, the cited art, *i.e.*, Demkow, does not anticipate the claimed invention. Applicants respectfully request reconsideration and withdrawal of the 35 U.S.C. §102(b) anticipation rejection in view of the claim amendments and above arguments.

Dependent Claims

The applicants have not independently addressed all of the rejections of the dependent claims. The applicants submit that for at least similar reasons as to why independent claim(s) 1 from which all of the dependent claims 2 and 3 depend are believed allowable as discussed *supra*, the dependent claims are also allowable. The applicants however, reserve the right to address any individual rejections of the dependent claims and present independent bases for allowance for the dependent claims should such be necessary or appropriate.

Thus, applicants respectfully submit that the invention as recited in the claims as presented herein is allowable over the art of record, and respectfully request that the respective rejections be withdrawn.

CONCLUSION

Based on the foregoing amendments and remarks, Applicants respectfully request reconsideration and withdrawal of the rejection of claims and allowance of this application. In the event that a telephone conference would facilitate examination of this application in any way,

the Examiner is invited to contact the undersigned at the number provided. Favorable action by the Examiner is earnestly solicited.

AUTHORIZATION

The Commissioner is hereby authorized to charge any additional fees which may be required for consideration of this Amendment to Deposit Account No. **13-4500**, Order No. 4439-4028.

In the event that an extension of time is required, or which may be required in addition to that requested in a petition for an extension of time, the Commissioner is requested to grant a petition for that extension of time which is required to make this response timely and is hereby authorized to charge any fee for such an extension of time or credit any overpayment for an extension of time to Deposit Account No. **13-4500**, Order No. 4439-4028.

Respectfully submitted,
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EXHIBIT 1

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Review Article

Antiangiogenesis in cancer therapy—endostatin and its mechanisms of action

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Abstract

The first angiogenesis inhibitors for cancer have now been approved by the F.D.A. in the U.S. and in 28 other countries, including China. The majority of these are monotherapies that block VEGF. However, mutant tumor cells may over time produce redundant angiogenic factors. Therefore, for long-term use in cancer, combinations of angiogenesis inhibitors or broad spectrum angiogenesis inhibitors will be needed. The two most broad spectrum and least toxic angiogenesis inhibitors are Caplostatin and endostatin. Endostatin inhibits 65 different tumor types and modifies 12% of the human genome to downregulate pathological angiogenesis without side-effects. The recent discovery that small increases in circulating endostatin can suppress tumor growth and that orally available small molecules can increase endostatin in the plasma suggests the possible development of a new pharmaceutical field.

Keywords: Endostatin; Angiogenesis inhibitors

Contents

Introduction

Angiogenesis inhibitors for the treatment of cancer have now been approved by the F.D.A. in the U.S. and in 28 other countries, including the European Union (Table 1). In December 2003, when thalidomide was approved in Australia for the treatment of multiple myeloma, Gareth Morgan, Chair of the U.K. Myeloma Forum Scientific Subcommittee, said “It is the best treatment advance in 25 years and people are doing well with it” [1]. When Avastin

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Table 1
Angiogenesis inhibitors approved by the FDA in the U.S. and in 28 other countries (including endostatin (China) and Macugen (U.S. and Brazil)

Date approved	Drug	Place	Disease
December 2003	Thalidomide	Australia	Multiple myeloma
February 2004	Avastin	U.S. (FDA)	Colorectal cancer
November 2004	Tarceva	U.S. (FDA)	Lung cancer
December 2004	Avastin	Switzerland	Colorectal cancer
December 2004	Macugen	U.S. (FDA)	Macular degeneration
January 2005	Avastin	European Union (25 countries)	Colorectal cancer
September 2005	Endostatin (Endostar)	China (SFDA)	Lung cancer

was approved in the U.S., for the treatment of colorectal cancer in February 2004, Mark McClellan, the Director of the FDA, stated that, "Angiogenesis inhibitors can now be considered as the fourth modality of cancer therapy." In May 2004, Andrew C. von Eschenbach, Director of the National Cancer Institute, and Allen M. Spiegel, Director of the National Institute of Diabetes and Digestive and Kidney Disease, said, "We can now unequivocally say that angiogenesis is not only a critical factor of cancer, but for a host of other diseases" [2]. In September 2005, endostatin (Endostar) was approved by the State FDA in China for the treatment of non-small-cell lung cancer [3]. In addition, other drugs previously approved by the FDA for other uses have recently been found to have antiangiogenic activity. These include doxycycline [4,5], zolendronate [6], rosiglitazone [7], celecoxcib [8], and low-dose interferon alpha [9,10] Furthermore, certain other anti-cancer drugs that were originally designed to inhibit a growth factor receptor, (i.e., Tarceva [11]), or to inhibit proteosomes (i.e., Velcade [12]), have also shown various degrees of antiangiogenic activity. At least 30–40 angiogenesis inhibitors are currently in preclinical or clinical trials. In addition to cancer therapy, the FDA recently approved Macugen [13] (pegaptanib), an aptamer of VEGF, for the treatment of macular degeneration.

Therefore, after more than three decades of research on angiogenesis, the translation of antiangiogenic therapy from laboratory to the clinic is underway and is robust. It is now possible to think about new opportunities which this novel class of drugs may permit for the treatment of cancer and other angiogenesis-dependent diseases. We can also ask about new directions in research that may further improve antiangiogenic therapy in the future.

Antiangiogenic monotherapy

The first FDA-approved angiogenesis inhibitor, Avastin (bevacizumab), blocks a single angiogenic protein, VEGF, produced by about 60% of human tumors. It was the first angiogenesis inhibitor to demonstrate significant prolongation of survival in colon cancer [14]. Significant increase in survival has also been demonstrated for lung cancer and breast cancer by antiangiogenic therapy (personal communication, Dr. Roy Herbst, in a Presidential Symposium on Angiogenesis at the American Society of Oncology, May 18, 2005).

However, advanced stages of breast cancer can express up to six proangiogenic proteins [15]. In advanced stages of neuroblastoma, high expression of seven angiogenic proteins is found [16]. Human prostate cancer can express at least four angiogenic proteins, including VEGF, bFGF, IL-8 and PDGF [17]. If these findings extend to other human cancers, as survival increases, it is likely that residual tumors in a given patient could express redundant angiogenic factors. Such tumors could become refractory to an angiogenesis inhibitor that blocks a single angiogenic factor. The result would simulate "acquired drug resistance" of tumor cells to cytotoxic chemotherapy. Other hypothetical mechanisms for resistance to antiangiogenic therapy are discussed in [18]. However, the possibility that a tumor could, over time, produce redundant angiogenic factors not matched by the angiogenesis inhibitor may already be problematic. Therefore, "resistance" to antiangiogenic therapy differs from tumor resistance to cytotoxic chemotherapy and may be preventable.

To prevent such a potential refractory state, combinations of angiogenesis inhibitors are already being used to broaden their therapeutic efficacy. These approaches include Avastin plus Tarceva or Avastin plus antiangiogenic chemotherapy [19] (metronomic chemotherapy) [20]. Furthermore, another class of angiogenesis inhibitors (i.e., Sugen11248 [21]) that can counteract three angiogenic proteins, VEGF, bFGF and TGF-alpha, is currently in clinical trial.

In the coming years, however, it is likely that *very broad spectrum* antiangiogenic therapy will be desirable, especially to facilitate a goal of "converting cancer to a chronic manageable disease" [22].

Broad spectrum antiangiogenic therapy

But, there are obstacles to the development of drugs with a broad spectrum of antiangiogenic activity. The conventional wisdom about a cytotoxic chemotherapeutic drug is that the more narrowly focused its target (i.e., "smart drug"), the more likely that the drug will be more active against a tumor and less toxic to normal tissues. Gleevec illustrates this concept, but it also reveals that a narrow target may lead to rapid onset of drug resistance. In contrast, there is a widely held belief that 'broad-spectrum' anti-

cancer drugs with 'multiple targets' will generate many side-effects. This may be true for cytotoxic anti-cancer drugs, but not for certain angiogenesis inhibitors.

For example, of the *synthetic* angiogenesis inhibitors, TNP-470 [23], a synthetic analogue of fumagillin, has an anti-cancer spectrum in pre-clinical studies that is broader than virtually any other anti-cancer drug. When TNP-470 is conjugated to a co-polymer (HPMA) to form Caplostatin [24], it has little if any toxicity over a broad range of effective doses.

Of the *endogenous* angiogenesis inhibitors in the body, endostatin has the broadest anti-cancer spectrum. It targets angiogenesis regulatory genes on more than 12% of the human genome [25], and yet it is the least toxic anti-cancer drug in mice. In humans, endostatin has virtually no toxicity and has revealed no resistance even when it has been administered to patients every day for up to >3.5 years without interruption. Endostatin, therefore, represents a model of a broad spectrum angiogenesis inhibitor that in the future could be a platform anti-cancer agent for co-administration with other therapies. The most important contribution of endostatin may be that it could introduce a shift in conventional thinking from the development of angiogenesis inhibitors for narrowly focused targets, toward angiogenesis inhibitors with multiple angiogenesis regulatory targets, but little or no toxicity. These two classes of angiogenesis inhibitors, 'focused target' and 'broad spectrum target', are not mutually exclusive and may be administered together. Because endostatin has recently been approved in China for non-small-cell lung cancer (Endostar), we may soon learn from the Chinese if this concept is validated.

Finally, one of many unexpected outcomes of the study of endostatin by hundreds of investigators (in 770 reports at this writing), over the 8 years since the first report of its discovery in 1997 [28], is that it can be elevated in the blood by small, orally available molecules (see below). This reveals the possibility of the emergence of an entirely new branch of pharmaceutics in which small, orally available molecules are developed to increase circulating levels of one or more of the 28 known endogenous angiogenesis inhibitors in the body [26,27]. In this paper, I will outline this new concept and the evidence for it. Endostatin is a centerpiece of this argument because more research has been published on this protein than on any other endogenous angiogenesis inhibitor.

Discovery of endostatin

Endostatin [28,29] is a 20 kDa internal fragment of the carboxyterminus of collagen XVIII [30,31]. It was discovered by Michael O'Reilly in the Folkman laboratory based on Folkman's hypothesis of a mechanism to explain the phenomenon that surgical removal of certain tumors leads to rapid growth of remote metastases. This hypothesis also initiated the prior discovery of angiostatin [32] in the same laboratory. In its simplest terms, this hypothesis said that "if tumors produce both stimulators and inhibitors of angiogenesis, the stimulators (i.e., VEGF, bFGF) could accumulate in excess of inhibitors *within* an angiogenic tumor. In the *circulation* however, the ratio would be reversed. Angiogenesis inhibitors would increase relative to stimulators, because of rapid clearance of stimulators from the blood." (VEGF has a half-life of ~3.5 min in the circulation.) Folkman formulated this hypothesis after reading Noel Bouck's first report in 1989 that the emergence of tumor angiogenesis was the result of a shift in balance between positive and negative regulators of angiogenesis in a tumor [33]. Endostatin is the first endogenous inhibitor of angiogenesis to be identified in a matrix protein.

Early problems

(a) Difficulties with production

Endostatin protein was initially purified from the urine of tumor-bearing mice, providing a few micrograms for analysis of amino acid sequence [28]. Recombinant mouse endostatin was then produced in *E. coli*. Endotoxin was removed by polymyxin chromatography. However, resolubilization methods at the time gave very low yields of active protein (~1–2%) that were insufficient for testing antiangiogenic and antitumor activity in vivo, but insoluble purified endostatin had the consistency of toothpaste. To overcome this impasse, insoluble endostatin was injected subcutaneously in mice. A white deposit formed (of approximately 2–3 mm diameter) that slowly disappeared over 2–3 days. The antitumor activity was dramatic. Murine tumors could be completely regressed as long as the mice received a daily injection of the insoluble endostatin [29]. Furthermore, discontinuation of endostatin therapy was followed by recurrence of tumor growth, but tumors could be completely and repeatedly regressed by resumption of endostatin therapy. This result demonstrated absence of acquired drug resistance and absence of toxicity, even when therapy was continued for more than 100 days. A surprising result in three out of three different tumor types was that, after prolonged endostatin therapy, tumors did not recur but remained dormant at a microscopic size throughout the normal lifetime of the mice. The mechanism of this sustained dormancy is unclear. There was general criticism of the insoluble endostatin experiments by reviewers and skeptical colleagues, who felt that insoluble endostatin protein was denatured. They ascribed the antitumor activity to contamination with bacterial endotoxin, despite the fact that the preparations were endotoxin-free.

(b) Difficulties in reproduction

When other laboratories tried to make their own recombinant endostatin from *E. coli*, there were incon-

sistencies of endostatin activity [34]. Furthermore, when the Folkman laboratory mailed active endostatin, *E.-coli*-derived preparations to colleagues, the insoluble *E. coli* preparations were not always active. Recently, Kashi Javaherian and Robert Tjin in the Folkman laboratory reported that "the entire antitumor, antiendothelial migration, and antipermeability activities of endostatin are mimicked by a 27-amino-acid peptide corresponding to the NH_2-terminal domain of endostatin" [35]. It is acid-resistant. Others have previously reported endostatin peptides [36–40].

"Aggregation of endostatin in *E. coli* preparations is caused by random intermolecular disulfides after PBS dialysis." While endostatin reveals "a single protein molecule under reducing conditions, most of the protein in an identical sample does not enter the polyacrylamide gel under nonreducing conditions. It is probably the degree of nonspecific aggregation that is responsible for the lack of activity in some of the earlier *E. coli* preparations" [35]. In animals, endostatin is most likely released in a sustained manner from the subcutaneously injected aggregate, resulting in "a denatured protein or partial fragments," which have antitumor activity due to their NH_2-terminal peptide. Some of the early *E. coli* preparations yielded larger aggregates which were inefficiently released or were inactive. This problem was solved by production of soluble endostatin (human and murine) in yeast (*Pichia pastoris*). Currently, virtually all laboratories around the world now produce their own recombinant endostatin from yeast or have used Entremed's soluble human recombinant endostatin from yeast.

However, Gorelik [41] obtained the *E. coli* plasmid from the Folkman laboratory, generated soluble endostatin at a yield of 150 mg/l and 99% purity and solubilized it by refolding the protein. He treated Lewis lung carcinomas with 20 mg/kg/day and obtained >99% tumor inhibition and also complete regression, thus reproducing O'Reilly and Folkman's original study with *E. coli* endostatin [28,29]. Furthermore, Perletti et al. in Milan purified rat endostatin from *E. coli* and treated spontaneous rat mammary carcinoma induced by a carcinogen [42]. Tumor regression was complete and yielded the same residual microscopic dormant nodules observed by O'Reilly and Folkman [28,29]. Furthermore, human recombinant endostatin recently approved in China [3] for the treatment of lung cancer is made from *E. coli* and is refolded to achieve a soluble product.

Soluble recombinant endostatin from yeast showed significant antiangiogenic and antitumor activity in mice but did not induce the complete tumor regressions previously observed with insoluble *E. coli* preparations, unless soluble endostatin was administered *continuously* by an implanted micro-osmotic pump [44,45] (Fig. 1). These results emphasized the importance of continuously elevated circulating levels of endostatin to achieve *optimum inhibition and regression of tumors*.

(c) The zinc controversy

Another problem was whether zinc binding by endostatin is necessary for its antiangiogenic and antitumor activity. A year after the first report of endostatin, Thomas Boehm in the Folkman laboratory showed that replacement of histidines 1 and 3 by alanines blocked the antitumor activity of endostatin [46]. This finding was challenged by two later reports [47,48]. In one report [47], a mutant endostatin was prepared by deleting five amino acids in the COOH and NH_2-termini. This construct appeared to have the same antitumor activity as full-length endostatin. However, in the renal cell carcinoma model employed, endostatin was administered only at the periphery of the tumor, and the injection dosage was only 10 micrograms/kg/day for 4 days. Endostatin was administered when the tumor size was 300 mm^3 and lasted for only 4 days when the tumor size reached 500 mm^3. In contrast, in our experiments, endostatin was administered systemically and was not injected into the periphery of the tumor. We initiated treatment of Lewis lung carcinoma at 100 mm^3 and continued until the untreated controls were ~6000 to 7000 mm^3. In another report [48], removal of 4 amino acids HSHR, from the NH_2-terminus, did not affect its antitumor activity. Measurements of Zn binding revealed that this mutant bound 2 atoms of Zn per molecule of

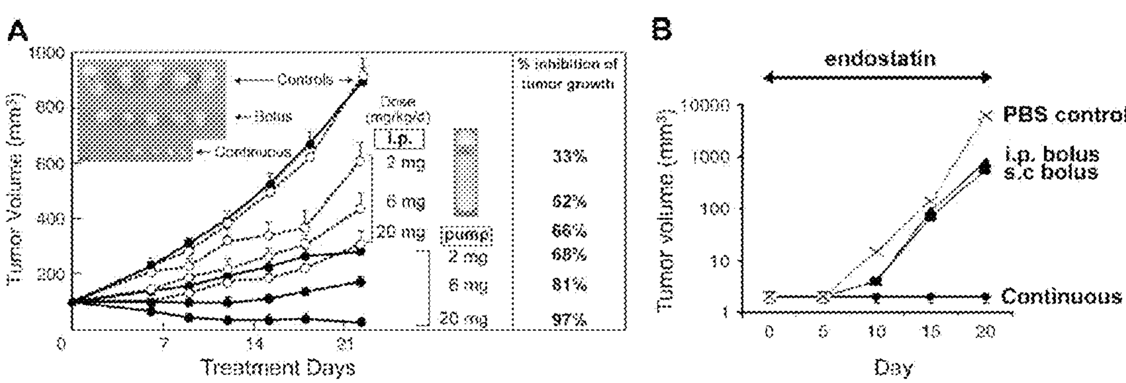

Fig. 1. Continuous administration of endostatin provides more effective antitumor activity than repeated bolus doses [45,44].

endostatin, whereas the wild-type bound 10 atoms of Zn per endostatin molecule. This finding is problematic because, in our crystal structure analysis of endostatin, the molecule contains one atom of zinc/endostatin molecule, and the removal of the four amino acids HSHR from the NH_2-terminus results in loss of zinc binding [49]. Other reports of peptides of endostatin that do not bind zinc, but were not tested against tumors, or were not compared at equimolar concentrations are discussed in [35], but not here to save space. The definitive experiments by Tjin and Javaherian [35] show that the 27-amino-acid peptide corresponding to the NH_2-terminal domain of endostatin contains three histidines that are responsible for zinc binding. Mutations of the zinc-binding histidines abolished its antitumor and antiendothelial migration activities, but not its antipermeability activity.

Because endostatin is generated by proteolytic cleavage of collagen XVIII [50,51], the first amino acid at the NH_2-terminus of endostatin is a histidine. Because the presence of histidine confers zinc binding to endostatin, we conclude that the processing of endostatin may be highly regulated.

Table 2A
Tumors significantly inhibited by recombinant endostatin protein

Tumor	Reference
Murine tumors	
Ovarian	[52]
Acute myelogenous leukemia (chloroma)	[53]
Colorectal carcinoma	[54]
Spontaneous mammary carcinoma	[56]
B-16 melanoma	[60]
Transplantable mammary carcinoma	[62]
Hepatoma	[101]
Lung adenocarcinoma	[70]
Lewis lung carcinoma	[41]
Rat glioma in brain (continuous endostatin + PKC)	[102]
Murine colorectal liver metastases	[103]
B-16 melanoma + endostatin fusion with angiostatin	[104]
Human myeloid leukemia in SCID rats	[105]
Lewis lung carcinoma	[106]
Rat carcinogen induced mammary cancer	[42]
Pancreatic insulinoma	[107]
Human tumors in mice	
Laryngeal squamous cell carcinoma	[55]
Glioblastoma (U87)	[57]
Prostate carcinoma (PC3)	[59]
Neuroblastoma	[61]
Testicular carcinoma	[64]
Breast carcinoma	[65]
Head and neck squamous cell carcinoma	[66]
Kaposi's sarcoma	[67]
Pancreatic carcinoma	[68]
Human non-small-cell lung cancer	[108]
Human pancreatic carcinoma	[45]
Brain tumors (U87)	[109]
Non-Hodgkin lymphoma (high grade)	[110]
Renal cell cancer	[111]
Bladder cancer	[112]
Murine metastatic tumors	
Lung adenocarcinoma (completely inhibited)	[70]

A few are discussed in detail in the text.

Antitumor activity by endostatin protein

Many animal and human tumors in mice have been inhibited by administration of endostatin protein in studies from different investigators (summarized in Table 2A). A few reports selected from more than 100 which show significant antitumor activity by endostatin are summarized below in order to illustrate certain principles of endostatin's therapeutic use. These examples also demonstrate some of the different types of murine and human tumors treated, as well as the different doses, schedules and routes of administration, employed by different investigators. All reports emphasize the lack of toxicity. Also endostatin does not interfere with wound healing. Tumor responses ranged from 47% to 91% inhibition by endostatin doses of 10 mg/kg to 100 mg/kg/day. Human ovarian cancer in nude athymic mice was inhibited by 73% by NGR-endostatin by day 41 compared to 58% inhibition of tumor growth with native endostatin administered at 20 mg/kg/day [52]. NGR-endostatin is a recombinant human endostatin (from yeast) genetically modified to contain an asparagine–glycine–arginine sequence (NGR) which is known to home to blood vessels because endothelial cells express high levels of aminopeptidase N. Murine ovarian cancer was also significantly inhibited.

A murine acute myelogenous leukemia (chloroma) in SCID mice was inhibited by 73% after 4 weeks by murine endostatin continuously released from microencapsulated cells transfected with murine recombinant endostatin (yeast) endostatin [53]. Survival was increased by 30% ($P \leq 0.003$), and microvessel density was significantly decreased. Endostatin in serum was 50 ng/ml in wild-type mice vs. 80 ng/ml in treated mice. Endostatin had no effect on the proliferation of tumor cells in vitro. In other mouse strains, serum endostatin levels have been reported as low as 10–15 ng/ml.

Liver metastases from murine colorectal cancer cells injected into the spleen were prevented by a 2 h pre-treatment in vivo with endostatin [54]. The dose of endostatin at 500 μg s.c. per day for 19 days is approximately equivalent to 25 mg/kg/day.

Human laryngeal squamous cell carcinoma in nude mice was inhibited by 45.9% ($P < 0.01$) at an endostatin dose of 20 mg/kg × 21 days. Intratumoral microvessel density was significantly decreased [55].

Spontaneous mammary carcinoma in transgenic mice was significantly inhibited by a mutated form of recombinant human endostatin [56].

Human brain tumors (U87) in a transparent chamber in the skulls of nude mice were inhibited by 74% (reduction in tumor volume) by direct microinfusion of endostatin for 3 weeks, at 2 mg/kg/day ($P = 0.05\%$). Microvessel density

was decreased by 33.5% ($P \leq 0.005$), and there was a 3-fold increase in tumor cell apoptosis ($P \leq 0.002$) [57]. Survival increased significantly ($P < 0.003$) and was dose-dependent.

In a model of human blood vascular cells which developed into hemangioma-like lesions in immunodeficient mice, endostatin inhibited these vascular lesions by 95% in 20 days [58]. Pericyte recruitment was inhibited by 35%.

Human prostate cancer (PC3), and human glioblastoma U87, in nude athymic mice at low dose (0.75 mg/kg/day) were administered endostatin q12 h for 14 days [59]. Tumor growth delay was significant for endostatin alone and for another angiogenesis inhibitor SU5416 and was increased 3- to 4-fold by both together ($P \leq 0.01$). This experiment illustrates the increased efficacy of broadening the spectrum of antiangiogenic activity. Functional vessel density was also decreased.

Murine melanoma (B16) in the foot pad was inhibited by 77% by human full-length endostatin, but only by 55% by a truncated human endostatin with deletion of N- and C-termini [60]. There was apparently no zinc binding in the truncated endostatin because of the absent histidine. However, lung metastases were inhibited more potently by the truncated endostatin than by full-length endostatin by i.p. injection of 0.3 mg/kg/day, after removal of a 1000 mm^3 tumor from the subcutaneous dorsum. These results suggest that zinc binding is not necessary for antitumor activity, but the peptide and the full-length endostatin were not tested at equimolar concentrations. Furthermore, it is not clear if the injection was intratumoral.

Human neuroblastoma was inhibited by 47% when human endostatin was administered subcutaneously at 10 mg/kg/day in nude mice for 10 days [61]. But, tumor inhibition was 61% when endostatin was administered continuously by an implanted pump at only 30% of the subcutaneous daily dose. These data further emphasize the improved efficacy from continuous delivery.

Murine mammary carcinoma implanted orthotopically was inhibited by endostatin administered at 50 mg/kg/day subcutaneously, and it synergized adriamycin without the increasing cardiotoxicity observed when other anti-cancer drugs are added to adriamycin [62].

Murine hepatoma was inhibited by subcutaneous administration of solubilized recombinant endostatin from *E. coli*. Microvessel density was significantly decreased, and tumor necrosis was increased [63].

Human testicular cancer was treated with endostatin administered at 10 mg/kg/day continuously by micro-osmotic pumps implanted subcutaneously.

Endostatin alone, carboplatin alone or thrombospondin-1 alone had *no* effect on the growth of the primary tumor or on metastases that occurred in all animals by 6 months [64]. However, a combination of endostatin plus thrombospondin-1, or a combination of endostatin plus carboplatin, *prevented all metastases*, significantly inhibited primary tumors, decreased vascularity, decreased tumor cell expression of VEGF-A and increased tumor cell apoptosis. These results emphasize another important principle: a tumor refractory to three drugs administered as single agents can become responsive to a combination of two angiogenesis inhibitors or to a combination of an angiogenesis inhibitor and a cytotoxic chemotherapeutic agent.

Human breast cancer in nude mice was inhibited by 80% when treated with a novel fusion protein of endostatin (at only 5 mg/kg/day) compared to 60% for endostatin alone [65]. This is another example that increasing the half-life of circulating endostatin increases its efficacy.

Human head and neck squamous cell carcinoma was significantly inhibited by endostatin therapy. However, endostatin also inhibited tumor cells directly by suppressing tumor cell migration and invasion, as well as by down-regulating gene expression of several pro-migratory molecules and upregulating AP-1 in the tumor cells. This is a first demonstration that, for some tumors, endostatin's clinical efficacy may extend beyond its antiangiogenic activity and include antitumorigenic activity as well, yet without toxicity to other tissues [66].

AIDS-related Kaposi's sarcoma is another example in which endostatin has antitumor and antiangiogenic activities. The tumor cells internalize endostatin which co-localizes to tropomysin microfilaments and inhibits cytokine-mediated migration and invasion of tumor cells [67].

Human pancreatic cancer in SCID mice was treated for 21 days by human recombinant endostatin administered subcutaneously at a relatively high dose (100 mg/kg/day) compared to other reports in the literature. A slowly growing pancreatic cancer (BXPC3) was inhibited by 91%. In contrast, the same dose inhibited the rapidly growing variant of this tumor (ASPC-1) by only 69% [68]. Endostatin significantly suppressed microvessel density by 66% ($P < 0.001$ for both tumors). This pair of tumors illustrates a general rule that slowly growing tumors are easier to treat with antiangiogenic therapy than are rapidly growing tumors, i.e., just the opposite of cytotoxic chemotherapy. This has also been reported for a pair of human bladder cancers one of which grows 10 times faster than the other [69]. For more rapidly growing tumors, higher doses of antiangiogenic therapy are generally required.

A murine lung adenocarcinoma (LA795) growing subcutaneously in mice was treated with endostatin at 20 mg/kg/day. The primary tumors were inhibited, and lung metastases were completely inhibited (100%), in contrast to control mice (PBS treated) with widespread metastases. Microvessel density was decreased ($P \leq 0.01$) [70].

There are three reports of lack of antitumor activity by endostatin protein. For example, a Morris hepatoma in rat liver did not respond to endostatin infusion into the hepatic artery, together with mitomycin C, plus lipiodol plus immunotherapy. The experiment was short-term and limited by the catheter life.

Antitumor therapy by endostatin gene therapy

Endostatin appears to be an ideal candidate for gene therapy. It is a highly conserved protein and in evolution is found as early as *C. elegans*. It has shown virtually no toxicity in animals or in patients, including four patients who have received endostatin daily for >3.5 years. In more than 60 reports since 1997, endostatin gene therapy of the full-length protein has significantly inhibited growth of primary tumors and their metastases. In animal studies, inhibition was up to 86% reduction in tumor volume and/or complete prevention of pulmonary metastases. Lowest inhibition of tumor growth was ~40–45% (Table 2B). In transgenic mice overexpressing endostatin, a small increase in circulating endostatin of approximately 1.6-fold is sufficient to confer dramatic protection against tumor growth [71]. In individuals with Down syndrome, a similar small increase of circulating endostatin is associated in part with broad protection against the majority of human tumors [72]. The recent report that all of the antitumor activity of endostatin is mediated by a 27-amino-acid N-terminal fragment of endostatin provides an expanded opportunity for future gene therapy with endostatin [35]. Below are representative examples of certain principles of endostatin gene therapy and of certain advantages over endostatin protein therapy (see also Table 2B).

When mouse endostatin was transfected into mouse renal cell carcinoma or human colon cancer cells so that endostatin was constitutively secreted, flank tumors were inhibited by 73–91% and liver and lung metastases were prevented or significantly inhibited [73]. Inoculation of a cell mixture containing only 25% endostatin-transfected tumor cells with 75% control tumor cells inhibited growth of flank tumors as effectively as 100% of endostatin-transfected cells [73]. This result suggests that "gene delivery of endostatin into even a minority of tumor cells may be an effective strategy to prevent progression of micrometastases to macroscopic disease."

Mouse brain tumor cells (C6 glioma) transfected with endostatin resulted in 71% inhibition of growth of orthotopic brain tumors implanted into brains of nude immunodeficient mice or in rats accompanied by a 50% decrease in microvessel density [74]. Complete tumor inhibition or dormancy was not observed in these mice. These results suggest that "endostatin could be developed as an adjuvant gene therapy for the treatment of brain tumors."

However, systemically administered antiangiogenic therapy may need to accompany endostatin gene therapy (for example, Caplostatin [75]).

In contrast to endostatin gene therapy alone, when endostatin gene therapy was combined with intratumoral adenovirus-mediated herpes simplex virus thymidine kinase, a cytotoxic virus, orthotopic renal cell cancer remained permanently dormant and was eradicated in 57% of treated mice [76]. This result indicates that cytotoxic gene therapy may be synergized by endostatin gene therapy.

When mouse breast cancer was implanted orthotopically into the mammary fat pad, it metastasized to brain. Intramuscular endostatin gene therapy increased the circulating levels of endostatin from the normal of 5 ng/ml (4.5–6.4 ng/ml) to a peak of 17.8 ng/ml (14.5–20.7 ng/ml) and inhibited the brain metastases by ~60%, but it had no effect on growth of the primary breast cancer [77]. This study shows that a rise in circulating endostatin level of only 3.5-fold is sufficient to inhibit growth of brain metastasis, but not the primary tumor. This relatively low level of increased endostatin reveals a differential effect on the primary vs. its metastasis that may present when endostatin therapeutic levels are borderline.

In a remarkable study of gene therapy of human glioblastoma xenografts in nude mice, a combination of 3 angiogenesis inhibitors was administered by intratumoral injection of plasmids containing two constructs, an angiostatin-endostatin fusion gene (statin-AE) and a soluble vascular endothelial growth factor receptor (sFlt-1) [78]. There was significant reduction in tumor microvessel density. Tumors were eliminated in up to 50% of mice (P = 0.003). Survival was prolonged by up to 4-fold (P = 0.008). Fifty percent of mice were still living at the end of the experiment (200 days). Intracranial and subcutaneous tumors were both successfully treated. These results show the advantage of combinatorial antiangiogenic gene therapy, especially for brain tumors. They also illustrate the advantage of intratumoral antiangiogenic gene therapy over systemic administration, at least for localized brain tumors, because gene transfer can facilitate sustained levels of inhibitor at the tumor site. Intratumoral antiangiogenic therapy of brain tumors may also produce a reverse diffusion of inhibitor toward neighboring capillaries.

Endostatin gene therapy enhanced the effect of ionizing radiation in Lewis lung carcinomas [79]. Tumor volumes were up to 50% smaller with the combination therapy. These results point to a possible future role for antiangiogenic gene therapy as a potentiator of ionizing radiation.

Endostatin gene therapy also enhanced the antitumor effect of gemcitabine and produced a significant decrease of tumor volume and of vascularization without added toxicity in a human lung cancer model in mice [80].

Several novel approaches for administering endostatin gene therapy have been reported. Intra-arterial delivery of endostatin gene therapy to rat brain tumors resulted in an 80% reduction in tumor volume, an enhanced survival time up to 47%, and a 40% decrease in number of tumor vessels [81]. Oral delivery of endostatin gene therapy by a unique bacterial carrier inhibited liver tumors in mice [82]. In certain colon cancers (murine C51, human HT29), endostatin directly inhibited the tumor cells in addition to its antiangiogenic activity.

Table 2B
Tumors significantly inhibited by endostatin gene therapy

Tumor	Reference
Murine primary tumors	
Renal cell carcinoma	[73]
Renal cell carcinoma	[113]
Brain tumors	[74]
Renal cell carcinoma	[114]
Renal cell carcinoma	[76]
Breast cancer and brain tumor (FM3A P-15) metastasis	[77]
Breast cancer (mid-T2-1)	[115]
Breast cancer (spontaneous)	[116]
Lewis lung carcinoma	[117]
Lewis lung carcinoma	[119]
Lewis lung carcinoma	[120]
Lewis lung carcinoma	[114]
Leukemia (L1210)	[113]
Myeloproliferative disease (resembling human chronic myelogenous leukemia)	[121]
Melanoma (K1735)	[122]
Melanoma (B16F10)	[123]
Bladder MBT-2	[123]
Colon cancer (colon 26)	[124]
Colon adenocarcinoma MC38	[125]
Hepatocarcinoma (H22)	[126]
Hepatoma (Hepa1c1c7)	[127]
Hepatocarcinoma	[101]
Melanoma (B16F10) (and metastases)	[128]
Spontaneous tongue carcinoma	[129]
Spontaneous breast cancer in C3(1)/T mice	[56]
Mammary carcinoma MCa-4	[130]
Brain tumor	[131]
Murine mammary ascites (TA3)	[132]
Neuroblastoma NXS2	[133]
Murine pulmonary metastases	
Fibrosarcoma	[134]
Fibrosarcoma (NFsa Y83)	[135]
Melanoma (B16F10)	[136]
Rat tumors	
Morris hepatoma	[137]
Hepatoma (orthotopic)	[138]
Gliosarcoma (9L)	[81]
Osteosarcoma	[139]
Hamster	
Pancreatic cancer (orthotopic) and liver metastases	[126]
Human tumors in mice	
Colon cancer (SW620)	[73]
Colorectal cancer (HT29)	[140]
Colorectal cancer (HT29)	[141]
Colorectal	[142]
Colorectal advanced stage IV (T3N1M1)	[143]
Colorectal cancer (LoVo)	[144]
Glioblastoma	[145]
Lung cancer	[80]
Lewis lung carcinoma	[118]
Non-small-cell lung cancer (KNS 62) (and metastases)	[146]
Hepatocellular carcinoma	[80]
Hepatocellular carcinoma (BEL-7402)	[147]
Hepatocellular carcinoma	[148]
Hepatocellular carcinoma Hep3B	[149]
Hepatocellular carcinoma HepGH	[43]
Hepatocellular carcinoma HepG2	[82]
Hepatocellular carcinoma HepG2	[150]
Hepatocellular carcinoma	[151]
Hepatocellular carcinoma (SMMC7721)	[152]
Ovarian cancer	[153]
Ovarian carcinoma	[154]
Ovarian carcinoma (SKOV3)	[155]
Ovarian carcinoma (SkOV3)	[156]
Tongue squamous cell carcinoma	[157]
Bladder carcinoma (KU-7) orthotopic	[158]
Lack of inhibition of angiogenesis, tumor growth and/or metastases	
Murine primary tumors	
Fibrosarcoma T241	[84]
Murine lung cancer	[89]
Lewis lung carcinoma (weak antitumor activity)	[83]
Human tumors	
Acute lymphocytic leukemia	[86]
Breast cancer (MDA-MB-231) (minimal effect)	[115]
Neuroblastoma (SKNAS)	[87]

A few are discussed in detail in text.

In 6 reports, endostatin gene therapy failed to inhibit tumor growth (Table 2B). In Kuo et al. [83] (from the Folkman lab) and in Pawliuk et al. [84], one explanation is that the circulating endostatin levels were too high. Since these papers were published, it has been found that endostatin antiangiogenic and antitumor efficacy is biphasic and operates over a U-shaped curve [85] (Fig. 2). Circulating levels of endostatin that are too high or too low are inactive. The normal range of endostatin in mouse blood among a wide variety of reports is ~5–15 ng/ml. Effective therapeutic levels are up to ~80–450 ng/ml. Higher levels may be less effective. In the paper by Jouanneau et al. [87], a possible explanation for endostatin's failure is variable aggregation of recombinant endostatin from *E. coli*, as discussed by Tjin et al. [35]. Other explanations are suggested by Steele [88]. Cui et al. [89] reported the very unusual upregulation of VEGF secretion from tumor cells by endostatin, and this may have overcome the antiangiogenic activity of endostatin. Tumors from these cells became hypervascularized and grew more rapidly instead of regressing as Lewis lung carcinoma did in all previous reports. The mechanism is unknown, although incubation of the tumor cells with endostatin did not elicit VEGF secretion and incubation of the gene transfected tumor cells with antibody to endostatin failed to stop VEGF secretion (Fig. 2).

Indraccolo has written a very thoughtful review of antiangiogenic gene therapy [90].

Mechanisms of the antiangiogenic activity of endostatin

Endostatin was discovered by employing the same strategy that led to the earlier discovery of angiostatin

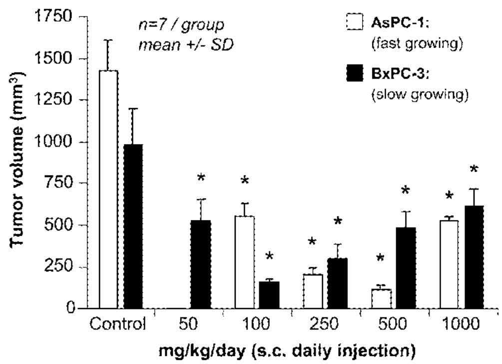

Fig. 2. Biphasic, U-shaped, dose response curve of endostatin. The dose and efficacy of certain endogenous (interferon alpha) and synthetic (rosiglitazone) angiogenesis inhibitors follow a biphasic, U-shaped relationship [85].

[91], i.e., isolation of an antiangiogenic protein from urine of tumor-bearing mice in which metastases are suppressed until the primary tumor is removed surgically. One of the major mechanisms of action of angiostatin, which explains most of its antiangiogenic functions, is based on its interaction with ATPase on the endothelial surface [92]. A second mechanism is angiomotin [93]. For endostatin, unlike angiostatin, a mechanism of action has not been distilled to a few signaling pathways. Since the first report of endostatin [28], numerous publications emphasize its broad spectrum anti-angiogenic mechanism of action. The clear picture to emerge is the pervasiveness of its influence—one that may be attributable in part to a long presence of the gene over the evolutionary course of the human genome. Several unique activities have been reported for endostatin since 1997. Endostatin binds alpha$_5$beta$_1$ integrin on endothelium [94,95]. For antiangiogenic activity, endostatin appears to be dependent on binding to E-selectin [96]. Also, endostatin blocks activity of metalloproteinases 2, 9, and 13 [97].

Shichiri and Hirata [98] showed that endostatin-initiated intracellular signaling in endothelial cells caused down-regulation of a set of growth-associated genes in a wide range of endothelial lineage cells. Abdollahi et al. [25,18] shed light on why the influence of endostatin is so extensive, and what this says about mechanism. Using custom microarrays covering over 90% of the human genome, they reported that ~12% of all genes are significantly regulated in human microvascular endothelial cells exposed to endostatin. They noted that the upregulated genes as a group include the known angiogenesis inhibitors, while the downregulated genes include the known stimulators. Revealed is a networked action of endostatin that cannot be reduced to single gene responses. To fully answer the question of mechanism, then it is necessary to think beyond individual molecular regulations and consider common physiological responses. On hundreds of genetic pathway fronts, endostatin is shown to suppress one physiological process—angiogenesis. From this perspective, it is not so surprising that the mechanism of endostatin has not been distilled to a few signaling cascades. More surprising is that there are so many genetic routes to angiogenesis suppression and that a single molecule can regulate these routes en masse to control, e.g., the angiogenic switch in tumors. In fact, a review of all published papers to date shows that endostatin suppresses mainly pathological angiogenesis and appears to have little or no activity against wound healing or reproduction. This phenomenon is not yet explained except for the possibility that certain pathological forms of angiogenesis are associated with upregulation of integrins (i.e., alpha$_5$beta$_1$) [71,94,95] or E-selectin [96] (Table 3).

Table 3
Selected genes associated with endostatin signaling (from[25])

Percent Down			Fold Up	
67%	VEGF-A		1.6	Maspin
72%	Neuropilin-1		1.7	DSCR5
66%	bFGF		6.0	IFN-g R1
44%	FGF-R1		2.7	Cdk4 inhib p18
30%	FGF-R2		2.2	Thrombospodin-1
55%	HGF		6.0	Sphingomyelinase
55%	EGFR		1.9	AT III
74%	HIF-1 alpha		2.0	Kininogen
60%	HIF1 Resp	Endostatin	2.8	STAT inh. (PIAS)
86%	Fibronectin		15	Notch 2
72%	Id1		4.0	Ephrin B3
60%	Id4		3.9	Ephrin A3
58%	c-myc		7.2	Collagen XVI a1
79%	c-fos		3.4	Adenomatosis Polyposis Coli
76%	Ephrin-A1		2.45	HIF-1α inhibitor (HIF1AN)
41%	NFKB p65		2.1	PDCD5 (Program cell death)
23%	bcl-2		2.7	SPOCK (sparc/osteonectin)
41%	TNF R-1			
31%	JUNB			
41%	cox-2			
63%	P-selectin			

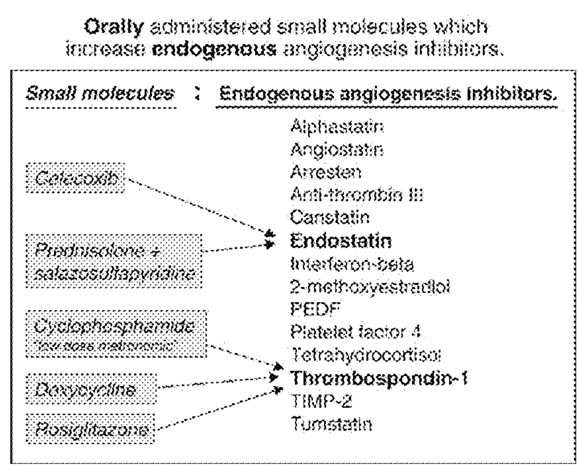

Fig. 3. Certain small molecules can induce elevations of endogenous angiogenesis inhibitors. These results suggest the possibility of a new pharmaceutical field.

Summary and future directions

At this writing, at least 28 endogenous angiogenesis inhibitors have been discovered in the plasma and/or in extracellular matrix [26,27]. Endostatin is the first endogenous angiogenesis inhibitor to be discovered as a fragment of the extracellular matrix. It is the most studied of the endogenous angiogenesis inhibitors. Kalluri showed that elevating the circulating level of endostatin (by genetic overexpression in endothelium) by less than 2-fold can suppress tumor growth by 2- to 3-fold [71]. It is possible that elevation of two or more endogenous inhibitors could possibly suppress tumor growth even more effectively or prevent it completely.

Several reports suggest that certain small molecules that can be taken orally will raise the endogenous expression of specific angiogenesis inhibitors or raise their plasma or serum level perhaps by alternative means, such as mobilization from matrix or platelets. For example, celecoxcib can increase serum endostatin [99]. Prednisolone and salazosulfapyridine can increase the endostatin level in joint fluid [100]. Doxycycline [5] and rosiglitazone can increase expression of thrombospondin-1. These are illustrated in Fig. 3. A possible new pharmaceutical field could be developed around the future discovery of low molecular weight, orally available drugs that could increase endogenous angiogenesis inhibitors to protect against cancer as well as other angiogenesis-dependent diseases. This would help to broaden antiangiogenic therapy of cancer. Endostatin is a paradigm of a broad spectrum endogenous antiangiogenic molecule.

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EXHIBIT 2

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A NOVEL EFFECT OF POLYMORPHONUCLEAR LEUKOCYTES IN THE FACILITATION OF ANGIOGENESIS

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Summary

The purpose of this study was to examine whether the adhesion of polymorphonuclear leukocytes (PMNs) to endothelial cells and/or reactive oxygen species (ROS) released from PMNs are responsible for inducing angiogenesis. Angiogenesis was assessed by tube formation using endothelial cells obtained from bovine thoracic aorta (BAECs) grown on a layer of collagen type I. Addition of PMNs to BAECs weakly induced angiogenesis. The angiogenesis induced by PMNs alone was further enhanced by treatment of the PMNs with N-formyl-methionyl-leucyl-phenylalanine (FMLP), a selective activator of PMN. The involvement of PMN adhesion to BAECs via adhesion molecules in angiogenesis was investigated by using monoclonal antibodies against E-selectin and intercellular adhesion molecule-1 (ICAM-1). These antibodies blocked both the PMN adhesion to BAECs and the enhancement of angiogenesis induced by FMLP-treated PMNs. Furthermore, the enhancement of angiogenesis by FMLP-treated PMNs was blocked by catalase, a scavenging enzyme of H_2O_2, but not by superoxide dismutase (SOD). These results suggest that PMNs induce angiogenesis in vitro, and that the mechanism of stimulation of angiogenesis by PMNs may involve the adherence of PMNs to endothelial cells via E-selectin and ICAM-1, and H_2O_2, but not superoxide. Thus, activated PMNs in pathological states may not only induce tissue injury, but may also function as regulators of angiogenesis.

Key Words: angiogenesis, PMNs, endothelial cells, H_2O_2, adhesion molecule

Angiogenesis, the formation of new blood vessels, occurs under various physiological conditions (1). Especially in inflammatory diseases such as wound healing, chronic

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inflammation, solid tumor formation, and diabetic retinopathy, it has been suggested that angiogenesis is involved in the maintenance of the inflammatory state by transporting inflammatory cells to the site of inflammation and supplying nutrients and oxygen to the inflamed tissue (2). In fact, the proliferating tissue contains an abundance of inflammatory cells, angiogenic blood vessels and inflammatory mediators in rheumatoid arthritis and the skin of psoriatic disease (3,4). Furthermore, it is reported that activated monocytes and macrophages are able to produce growth factors and cytokines which regulate angiogenesis (5,6). However, the role of neutrophils, another type of inflammatory cells in angiogenesis has not been fully evaluated. The recruitment of PMNs is believed to be one of the important mechanisms in the pathophysiology of various inflammatory diseases (7).

The adhesion and transmigration of PMNs to endothelial cells are mediated by adhesion molecules, such as E-selectin and ICAM-1, in inflammatory diseases (8-11). Recently, adhesion molecules have been reported to act as signaling receptors which mediate outside-in signaling in endothelial cells based on the fact that adherence of leukocytes or PMNs to endothelial cells through some adhesion molecules induces change in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of endothelial cells (12). On the other hand, it is suggested that Ca^{2+} entry into endothelial cells produces change in endothelial cell shape and initiates angiogenesis (13). Therefore, it is possible that adhesion of PMNs may stimulate angiogenesis.

Another function of activated PMNs during inflammation is the release of ROS, such as H_2O_2, superoxide anion ($O_2^{-}\cdot$) (14,15). Although relatively high concentrations of H_2O_2 ($\geqq 125$ μM) are known to induce endothelial cell injury (16), the concept of H_2O_2 action has gradually been changed due to reports that lower concentrations of H_2O_2 (25-100 μM) cause reversible alterations of signal transduction such as tyrosine and serine/threonine phosphorylations (17), and gene transcription such as NF-κB and AP-1 in endothelial cells (17,18). Moreover, we recently found that exogenously added H_2O_2 (0.1-10 μM) stimulates expression of a transcription factor, ets-1, which may cause an increase in collagenase production and upregulates angiogenesis in BAECs (19). However, it is unclear whether the H_2O_2 released from PMNs induces angiogenesis.

In the present study, we evaluated the involvement of PMN adhesion to endothelial cells via adhesion molecules and of ROS released from PMNs in the induction of angiogenesis by activated PMNs.

Materials and Methods

Cell culture

Bovine aortic endothelial cells were obtained by scraping the luminal surface with a razor blade, and cultured in monolayers as previously described (19). Cells at 3 to 8 passages were used for the experiments.

Preparation of PMNs

PMNs were collected from male Wistar rats (6-8 weeks old, Saitama Animal Supply Co. Ltd., Saitama, Japan). Briefly, each rat was injected intraperitoneally (i.p.) 5 ml of 0.5% oyster glycogen in saline. After 4 hrs, the rat was injected (i.p.) with 4 ml of 100 U/ml heparin and sacrificed. The cells infiltrated in the abdominal cavity were collected with 50 ml of PBS containing 10% fetal bovine serum (FBS). After centrifugation (170×g) for 10 min at 4°C, the supernatant was discarded and the remaining red pellet underwent hypotonic lysis by the addition of 0.2 % NaCl. After 30 seconds, the lysate underwent an isotonization by the addition of an equal volume of 1.6% NaCl solution, and centrifuged at 170×g for 10 min. The

supernatant was discarded and the residual pellet was washed twice with 10 ml of phosphate-buffered saline (PBS) containing 2% FBS and then centrifuged at 170×g for 10 min. The pellet was then suspended in 2 ml of minimum essential medium (MEM) containing 2% FBS. The purity of PMNs was confirmed by May Grünwald-Giemsa staining (>95%).

Tube formation

Tube formation was measured in 24-well culture plates using the three-dimensional culture method described in our previous report (19). Collagen gel solution (0.5 ml) consisting of a mixture of 8 volumes of type I collagen solution (KOKEN Co., Ltd. Tokyo, Japan), 1 volume of 10-fold concentrated MEM, 1 volume of 0.05 N NaOH, 200 mM HEPES, and 260 mM $NaHCO_3$, was poured into each well of the culture plates, and incubated for 60 min at 37 °C. BAECs suspended in 1 ml of MEM containing 10% FBS were added to the well and cultured. When the culture reached confluent, the medium was replaced with MEM containing 2% FBS and various concentrations of PMNs with or without FMLP (1 μM), and incubated for 3 days at 37 °C. Catalase (10 U/ml), SOD (50 U/ml), mouse anti-human ICAM-1 (CD54) monoclonal antibody (50 μg/ml) (IMMUNOTECH, Marseille, France), or mouse anti-human E-selectin (CD62E) monoclonal antibody (50 μg/ml) (PHARMIGEN, CA, U.S.A.) were added before 15-min of PMN treatment. The cultures were washed three times with PBS and fixed with 2.5% glutaraldehyde in PBS. Subsequently, randomly selected fields measuring 0.86 x 1.3 mm were photographed in each well under phase-contrast microscopy. Tube formation was quantitated from three randomly selected fields per experiment by measuring the total additive length of all cellular structures including all branches, using a computer-assisted image analyzer (MCID, Imaging Research Inc., Ontario, Canada).

PMN-BAECs adhesion assay

Cultures of BAECs were grown to confluence in 12-well plates, and the medium was changed to fresh MEM containing 2% FBS. PMNs (1×10^5 cells/well) were added to the cultures in the presence or absence of FMLP (1 μM) for 30 min. After incubation, non-attached cells were washed out three times with PBS containing 2% FBS, and 50 μl of citrate buffer (pH 4) containing 0.1% Triton X-100 was added to each well. After 5-10 min, 50 μl of *o*-phenylene diamine (OPDA)-citrate buffer solution containing 9.25 mM OPDA and 8.82 mM H_2O_2 was added to each well. After incubation at room temperature for 20-30 min, 50 μl/well of 4 N H_2SO_4 was added to stop the reaction, and the myeloperoxidase activity of PMNs was measured at OD_{490}.

Statistical analysis

Results are expressed as the means±SEM of n observations for each experiment. Statistical analysis was performed with the Bonferroni/Dunn procedure following ANOVA. Differences between means were considered significant at $p<0.05$.

Results

Effect of PMNs on angiogenesis

Typical morphological changes of BAECs are shown in Fig. 1. Under control conditions, BAECs grew to confluent monolayers in a cobblestone pattern on the surface of collagen gels (Fig. 1A). After addition of PMNs, BAECs invaded the underlying collagen gel to form a network of branching cellular cords beneath the surface monolayer, suggesting the induction of angiogenesis (Fig. 1B). Moreover, the angiogenesis induced by PMNs was significantly increased by simultaneous treatment with 1 μM FMLP (Fig. 1C). The effects of PMNs and

PMNs plus FMLP on angiogenesis shown in Fig. 1 are summarized in Fig. 2. Addition of 1×10^5 or 3×10^5 PMNs induced angiogenesis of BAECs, and PMNs stimulated by FMLP further enhanced the angiogenesis (Fig. 2). When more than 1×10^6 PMNs were added, the proliferation of the BAECs was inhibited, and BAEC lysis was observed in the three-dimensional cultures (data not shown). Addition of FMLP alone or the supernatant from PMNs to BAECs did not induce any morphological changes including angiogenesis (data not shown).

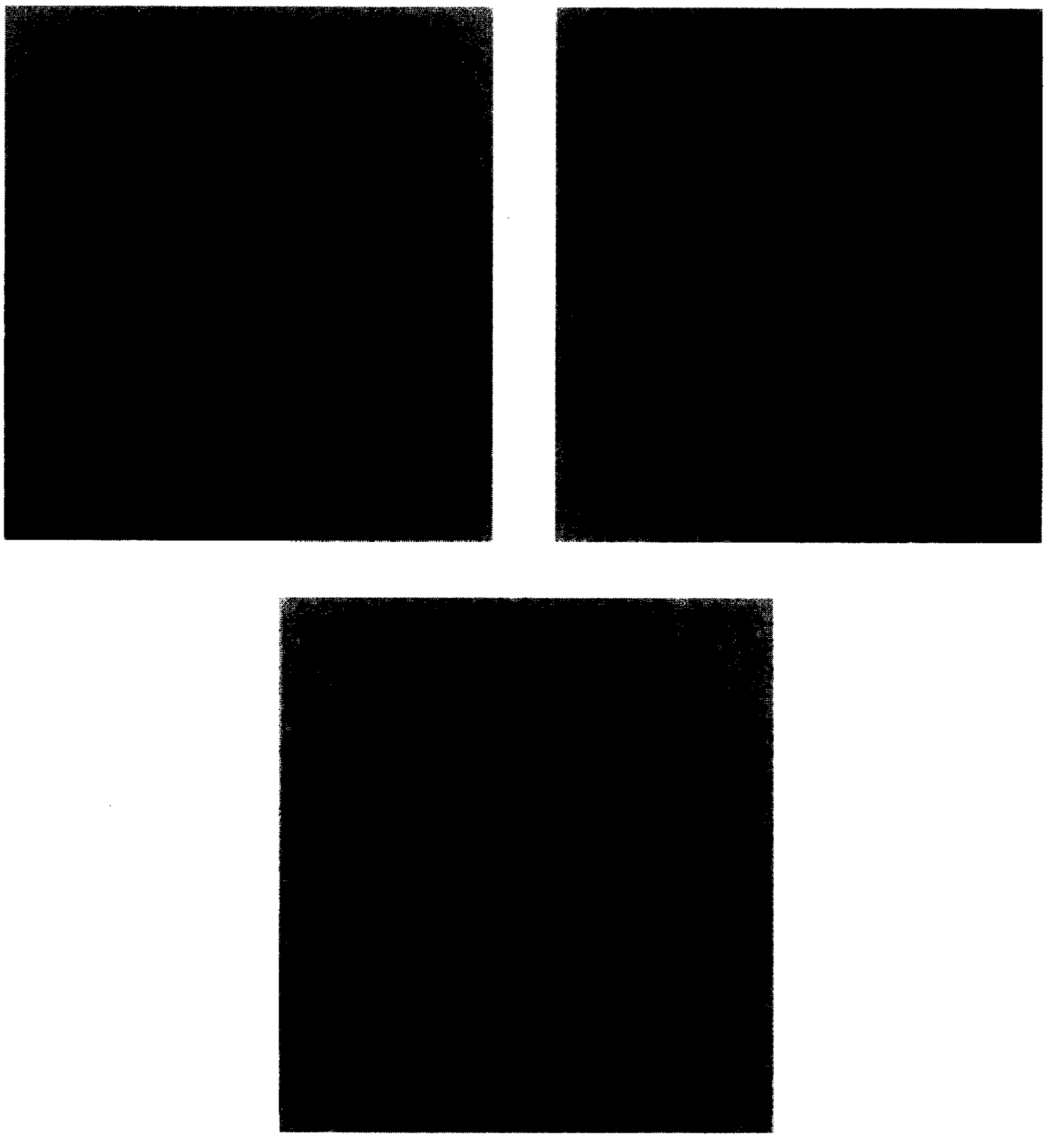

Fig. 1

Photomicrographs showing angiogenesis in co-cultures of PMNs with BAECs. Confluent monolayers of BAECs cultured on type I collagen gels were incubated with MEM containing 2% FBS as control (A), PMNs (1×10^5 cells/well) (B), or PMNs plus FMLP (1 μM) (C) in 24-well culture plates. After incubation for 3 days, the cultures were fixed with 0.25% glutaraldehyde and photographed. Original magnification, x100.

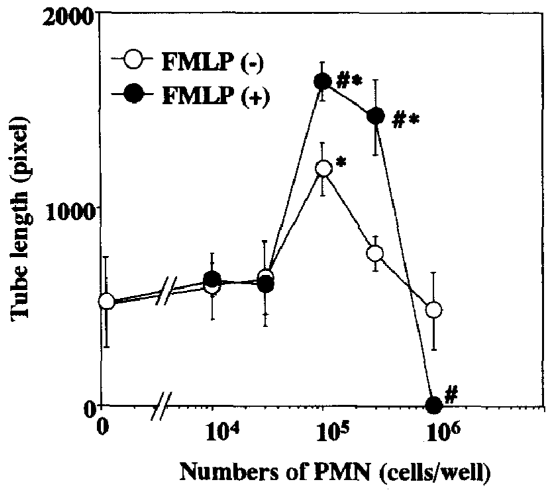

Fig. 2
Concentration-response curve of PMN-induced angiogenesis with or without FMLP stimulation. Confluent monolayers of BAECs cultured on type I collagen gels were co-cultured with PMNs with or without FMLP (1 μM) for 3 days. Results are expressed as the means ± SEM of 5 experiments with triplicate determinations in each assay. #P<0.05 *vs.* the value PMN without FMLP; * P <0.05 *vs.* the value without PMN and FMLP.

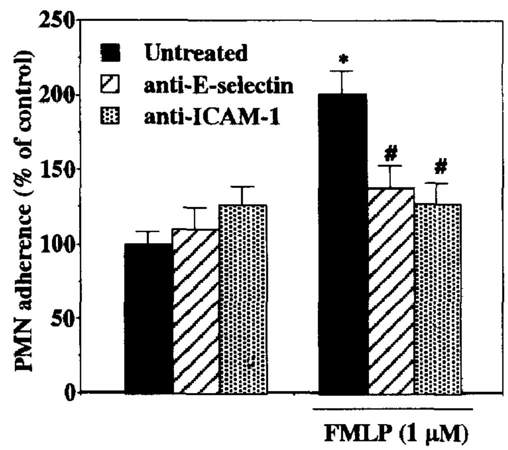

Fig. 3
Effects of anti-E-selectin and anti-ICAM-1 antibodies on adhesion of PMNs to BAECs. BAECs were preincubated with or without anti-E-selectin (5 μg/ml) or anti-ICAM-1 (5 μg/ml) monoclonal antibody for 30 min. PMNs ($1x10^5$ cells/well) treated with or without 1 μM FMLP were added to the BAECs. FMLP-treated or -untreated PMNs were then added to the BAECs. After 30-min of incubation, non-attached cells were washed out and adherent PMNs were quantified by measuring the myeloperoxidase activity. Results are expressed as the mean ± SEM of percent binding to BAECs relative to that of PMNs not treated with FMLP. The results are expressed as the mean ± SEM of 3 experiments with triplicate determinations in each assay. * P <0.05 *vs.* PMNs not treated with FMLP; # P <0.05 *vs.* FMLP-treated PMNs.

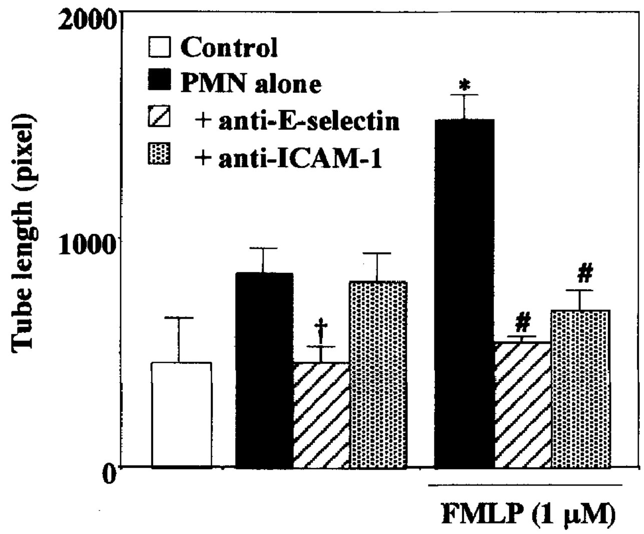

Fig. 4

Effects of anti-E-selectin and anti-ICAM-1 antibodies on PMN-induced angiogenesis. BAECs were preincubated with or without anti-E-selectin (5 μg/ml) or anti-ICAM-1 (5 μg/ml) monoclonal antibody for 30 min. FMLP (10^{-6} M)-treated or -untreated PMNs (1×10^5 cells/well) were then added to BAECs and incubated for 3 days. Results are expressed as the mean ± SEM of 3 experiments with triplicate determinations in each assay. * $P < 0.05$ *vs*. BAEC alone (control), # $P < 0.05$ *vs*. FMLP-treated PMNs, and †$P < 0.05$ *vs*. PMN not treated with FMLP.

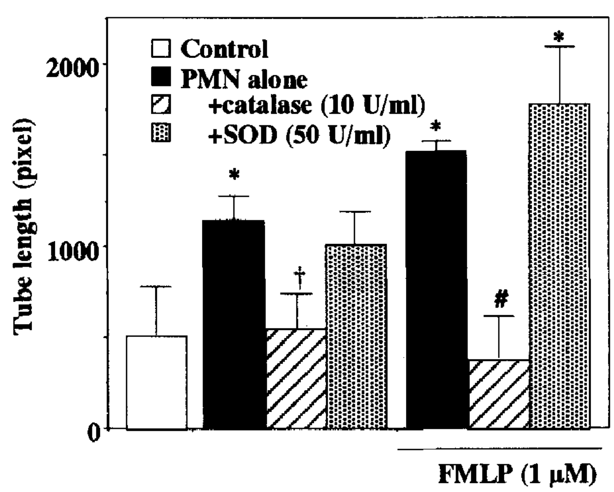

Fig. 5

Effects of catalase and SOD on angiogenesis induced by PMNs. BAECs were preincubated with 10 U/ml catalase or 50 U/ml SOD for 30 min, and were co-cultured with FMLP (1 μM)-treated or -untreated PMNs (1×10^5 cells/well) for 3 days. Results are expressed as the mean ± SEM of 3 experiments with triplicate determinations in each assay. * $P < 0.05$ *vs*. BAECs alone (control), # $P < 0.05$ *vs*. FMLP-treated PMNs, and †$P < 0.05$ vs. PMNs not treated with FMLP.

Involvement of adhesion molecules on adhesion of PMNs to BAECs

The adhesion between PMNs and BAECs was significantly enhanced by treatment with FMLP, as compared to the control (without FMLP) (Fig. 3). This enhancement by FMLP of PMN adhesion to BAECs was inhibited by pretreatment of BAECs with anti-E-selectin or anti-ICAM-1 antibody.

Involvement of adhesion molecules in PMN-induced angiogenesis

Addition of PMNs to BAECs induced angiogenesis, and the angiogenesis induced by PMNs was enhanced by FMLP. Anti-E-selectin antibody inhibited the angiogenesis induced by FMLP-treated and –untreated PMNs. On the other hand, anti-ICAM-1 antibody inhibited the angiogenesis induced by FMLP-treated PMNs, but not by PMNs not treated with FMLP (Fig. 4).

Effects of catalase and SOD on angiogenesis induced by PMNs

Treatment with 10 U/ml catalase completely inhibited the angiogenesis induced by FMLP-treated and –untreated PMNs (Fig. 5). However, 50 U/ml SOD did not affect the angiogenesis (Fig. 5).

Discussion

Angiogenesis is often observed in inflammatory states. In corneal inflammation, infiltrated PMNs are observed around the neovascular vessels (20-22). However, whether PMNs affect angiogenesis in the inflammatory states remains known. This study therefore examined the role of PMNs in angiogenesis in vitro.

Adhesion of PMNs to endothelial cells is known to trigger various physiological changes (8-11). Furthermore, it is also known that FMLP, a chemotactic factor, enhances the adhesion of PMNs to endothelial cells through adhesion molecules such as E-selectin and ICAM-1 (23). In the present study, we investigated the involvement of PMN adhesion in angiogenesis of BAECs. PMNs treated with FMLP had enhanced adhesion to BAECs as compared to PMNs not treated with FMLP, and the enhancement of adhesion was inhibited by anti-E-selectin and anti-ICAM-1 antibodies. Both anti-ICAM-1 and anti-E-selectin antibodies also inhibited the angiogenesis induced by FMLP-treated PMNs. These findings suggest that the enhancement of angiogenesis by PMNs in the presence of FMLP might involve cell-cell adhesion between PMNs and endothelial cells, and that the adhesion seems to be mediated by E-selectin and ICAM-1. On the other hand, angiogenesis induced by PMNs not treated with FMLP was inhibited by anti-E-selectin antibody, but not by anti-ICAM-1antibody. This result shows that angiogenesis induced by PMN alone might be mediated by E-selectin. However, we have not ruled out possibility that the inhibition of angiogenesis by anti-E-selectin antibody might be partly caused by direct inhibition of tube formation, in addition to inhibition of the adhesion of PMNs to endothelial cells. Indeed, there are reports that antibodies against E-selectin and its counter receptor, sialyl Lewis-X/A, inhibit capillary formation of serum-starved bovine capillary endothelial cells formed on fibronectin (24,25).

It has been reported that adhesion of PMNs to endothelial cells through adhesion molecules induces a $[Ca^{2+}]_i$ increase in endothelial cells (12). Moreover, $[Ca^{2+}]_i$ changes in endothelial cells have been reported to be involved in capillary formation (13). These results support our hypothesis that adhesion of PMNs to endothelial cells via adhesion molecules might stimulate angiogenesis.

It is recognized that the adhesion of PMNs to endothelial cells and the ROS released

from inflammatory cells, including PMNs, induce cell injury in inflammatory diseases. Recently, however, H_2O_2 (25-100 μM), a representative ROS, has been reported to cause reversible alterations of signal transduction and gene transcription in endothelial cells (17). Moreover, our previous report has revealed that relatively low concentrations of H_2O_2 (0.1-10 μM) induces angiogenesis by induction of the transcription factor ets-1, which regulates the gene expression of proteases such as urokinase-type plasminogen activator and matrix metalloproteinase-1 (19). Therefore, H_2O_2 seems to be not only a cytotoxic factor but also a physiological regulator of endothelial cell function. To clarify the relation between ROS and PMNs in the induction of angiogenesis, BAECs were treated with catalase or SOD. Catalase, but not SOD, inhibited the PMN-induced angiogenesis. It has been reported that PMNs treated with FMLP produce a few μM H_2O_2 per 3×10^5 cells for 3 min (26). These results suggest that H_2O_2, but not superoxide, released from PMNs plays an important role in angiogenesis.

In the present study, we showed that both adhesion molecules and H_2O_2 are important factors in the enhancement of angiogenesis by PMNs. Several studies have indicated that the localization of PMNs in inflammatory disease depends, in part, on intercellular adhesion, as shown by reduction of PMN accumulation or tissue injury after the treatment with monoclonal antibodies against the adhesion molecules (27), and leukocyte accumulation also appears to depend on ROS, as shown by the inhibitory effects of catalase and SOD (28). Moreover, H_2O_2 has been reported to stimulate the expression of adhesion molecules (29). These reports have suggested that it is possible that PMN-induced angiogenesis might be related to expression of adhesion molecules in endothelial cells following the release of H_2O_2 from PMNs. On the other hand, it has been reported that PMN adhesion to endothelial cells through ICAM-1 increases the intracellular H_2O_2 level in endothelial cells, and that the increases in H_2O_2 are blocked by anti-ICAM-1 antibody (30). An another possibility is that PMN adhesion to endothelial cells via ICAM-1 increases the H_2O_2 content in endothelial cells, and that this H_2O_2 might up-regulate angiogenesis. Future studies will be needed to examine the relationship between adhesion molecules and H_2O_2 in PMN-induced angiogenesis.

In conclusion, the findings in the present study demonstrated that PMNs stimulated angiogenesis in vitro, and that the induction mechanisms of angiogenesis might involve cell-cell adhesion and H_2O_2. Hence, PMNs may play a critical role in the initiation of angiogenesis in inflammatory diseases.

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EXHIBIT 3

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Differential roles of ICAM-1 and E-selectin in polymorphonuclear leukocyte-induced angiogenesis

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Yasuda, Masako, Shunichi Shimizu, Kyoko Ohhinata, Shinji Naito, Shogo Tokuyama, Yasuo Mori, Yuji Kiuchi, and Toshinori Yamamoto. Differential roles of ICAM-1 and E-selectin in polymorphonuclear leukocyte-induced angiogenesis. *Am J Physiol Cell Physiol* 282: C917–C925, 2002; 10.1152/ajpcell.00223.2001.—Ets-1, which stimulates metalloproteinase gene transcription, has a key role in angiogenesis. We first examined whether activated polymorphonuclear leukocytes (PMNs) enhanced angiogenesis through the induction of Ets-1. Addition of activated PMNs to endothelial cells stimulated both in vitro angiogenesis in collagen gel and Ets-1 expression. Both angiogenesis and Ets-1 expression induced by PMNs were reduced by *ets*-1 antisense oligonucleotide, suggesting that Ets-1 is an important factor in PMN-induced angiogenesis. Although intercellular adhesion molecule (ICAM)-1 and E-selectin are involved in PMN-induced angiogenesis, the mechanisms underlying their roles in angiogenesis have yet to be elucidated. PMN-induced Ets-1 expression was reduced by a monoclonal antibody against ICAM-1 but not E-selectin despite the inhibition of PMN-induced angiogenesis by both antibodies. Moreover, the stimulation of angiogenesis by H_2O_2 without PMNs was inhibited by a monoclonal antibody to E-selectin but not ICAM-1. These findings suggested that ICAM-1 in endothelial cells may act as a signaling receptor to induce Ets-1 expression, whereas E-selectin seems to function in the formation of tubelike structures in vascular endothelial cell cultures.

endothelial cell; intercellular adhesion molecule-1; Ets-1

ANGIOGENESIS, formation of new blood vessels, occurs under various pathological conditions (8). Especially in inflammatory diseases such as wound healing, chronic inflammation, solid tumor formation, and diabetic retinopathy, angiogenesis has been shown to be involved in maintenance of the inflammatory state by transporting inflammatory cells, nutrients, and oxygen to the site of inflammation (15). In fact, inflammatory tissue contains an abundance of inflammatory cells, angiogenic blood vessels, and inflammatory mediators (17, 18). Although the mechanisms of angiogenesis during inflammation remain unclear, monocytes and macrophages activated by inflammatory stimuli have been shown to induce angiogenesis through production of growth factors and cytokines (19, 33). In addition, we recently found (38) that activated polymorphonuclear leukocytes (PMNs) can also stimulate angiogenesis. Thus not only activated monocytes and macrophages but also activated PMNs seem to have important roles in stimulating angiogenesis in inflammatory diseases.

Ets-1 is a transcription factor that regulates the gene expression of proteases such as urokinase-type plasminogen activator (u-PA), matrix metalloproteinase (MMP)-1, MMP-3, and MMP-9 (11, 14, 27, 34). Many studies have shown that Ets-1 mediates angiogenesis. Iwasaka et al. (14) reported that vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) induce Ets-1 expression and Ets-1 stimulates angiogenesis by inducing the expression of u-PA and MMP-1. Moreover, Oda et al. (27) reported that overexpression of Ets-1 in vascular endothelial cells induced angiogenesis in vitro. Thus Ets-1 seems to play a central role in angiogenesis.

PMNs activated during inflammation adhere to endothelial cells (2, 36). The adherence of PMNs to endothelial cells is mediated by adhesion molecules such as E-selectin and ICAM-1 expressed in endothelial cells (9, 12, 39). We previously demonstrated (38) that ICAM-1 and E-selectin are involved in the induction of angiogenesis by PMNs because anti-ICAM-1 and anti-E-selectin antibodies inhibited PMN-induced angiogenesis. Recently, adhesion molecules have been reported to act as the signaling receptors that mediate changes in intracellular Ca^{2+} concentration (24) and tyrosine phosphorylation (5). Interestingly, the activation of tyrosine kinase has been reported to be involved in the induction of *ets*-1 in endothelial cells stimulated by VEGF (30). Therefore, it is possible that the signal transduction from adhesion molecules induces Ets-1 and then stimulates angiogenesis. Alternatively, adhesion molecules may have roles in cell-cell adhesion

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between endothelial cells in the process of PMN-induced angiogenesis. However, the roles of ICAM-1 and E-selectin in the process of PMN-induced angiogenesis have yet to be elucidated.

In the present study, we found the participation of Ets-1 in PMN-stimulated angiogenesis in bovine aortic endothelial cells (BAECs). Therefore, we investigated the roles of adhesion molecules in the induction of angiogenesis using Ets-1 expression and stimulation of angiogenesis with PMNs.

METHODS

Cell culture. BAECs were obtained by scraping the luminal surface with a razor blade and cultured as described previously (37). Endothelial cells were characterized by microscopic observation and incorporation of acetylated low-density lipoprotein labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (13). Cells at *passages 3–8* were used for the experiments.

Preparation of PMNs. PMNs were collected from male Wistar rats (6–8 wk old; Saitama Animal Supply, Saitama, Japan) as previously described (38). Each rat was injected intraperitoneally with 5 ml of 0.5% oyster glycogen in saline. After 4 h, the rats were injected intraperitoneally with 4 ml of 100 U/ml heparin. The cells infiltrating the abdominal cavity were collected with 50 ml of phosphate-buffered saline (PBS) containing 10% fetal bovine serum (FBS). After centrifugation (170 *g*) for 10 min at 4°C, the supernatant was discarded and the remaining red pellet was subjected to hypotonic lysis by addition of 0.2% NaCl. After 30 s, the lysate was made isotonic by addition of an equal volume of 1.6% NaCl solution and centrifuged at 170 *g* for 10 min. The supernatant was discarded, and the residual pellet was washed twice with 10 ml of PBS containing 0.1% FBS. The pellet was then suspended in 2 ml of minimum essential medium (MEM) containing 0.1% FBS. The purity of PMNs was confirmed by May Grünwald-Giemsa staining (>95%).

Tube formation assay. Tube formation was measured in 24-well culture plates with the three-dimensional culture method described in our previous report (38). Collagen gel solution (0.5 ml) consisting of a mixture of 8 volumes of type I collagen solution (Koken, Tokyo, Japan), 1 volume of 10-fold concentrated MEM, 1 volume of 0.05 N NaOH, 200 mM HEPES, and 260 mM $NaHCO_3$ was poured into each well of the culture plates and incubated for 60 min at 37°C. The BAEC suspension (5×10^5 cells/ml) in 1 ml of MEM containing 10% FBS was added to the wells and cultured. When the cultures reached confluence, the medium was replaced with MEM containing 0.1% FBS. After 48 h, various numbers of PMNs with or without 1 μM *N*-formylmethionyl-leucyl-phenylalanine (FMLP) were added and incubated for 3 days at 37°C. Mouse anti-human ICAM-1 (CD54) monoclonal antibody (50 μg/ml; Immunotech, Marseille, France) and mouse anti-human E-selectin (CD62E) monoclonal antibody (50 μg/ml; Pharmingen, San Diego, CA) were added 15 min before PMN treatment. The cultures were washed three times with PBS and fixed with 2.5% glutaraldehyde in PBS. Randomly selected fields measuring 0.86×1.3 mm were photographed in each well under phase-contrast microscopy. Tube formation was quantified from three randomly selected fields per experiment by measuring the total additive length of all cellular structures including all branches with a computer-assisted image analyzer (MCID; Imaging Research).

Diffusion chamber assay. To examine whether activated PMNs stimulate in vivo angiogenesis, we used a diffusion chamber assay system modified to assess in vivo angiogenesis as previously described (35). The diffusion chamber was made from a chamber kit purchased from Millipore (Bedford, MA). A cellulose membrane filter (0.45 μm, 14-mm diameter) was glued to each side of the ring chamber with MF (Millipore) cement. Male Wistar rats (200–250 g) were anesthetized by intraperitoneal injection of pentobarbital sodium (10 mg/rat). Before chamber implantation, the backs of the animals were depilated and disinfected with tincture of iodine. The chambers containing PMNs or vehicle were implanted into a subcutaneous pocket in the back of the rats. Seven days after implantation, the chambers were removed from the animals and fixed with 10% formalin solution.

Northern blot hybridization. BAECs were grown to 90% confluence in MEM containing 10% FBS and antibiotics, and then the cultures were starved in MEM containing 0.1% FBS for 48 h. PMNs stimulated with or without FMLP were added to the cultures and incubated for various periods. Total RNA was extracted from BAECs by a modified guanidinium isothiocyanate method with ISOGEN (Nippon Gene, Tokyo, Japan). Aliquots of 20 μg of total RNA were separated by electrophoresis through 1% agarose-formaldehyde gels. The RNA was transferred onto Hybond-N nylon membranes (Amersham Pharmacia Biotech, Little Chalfont, UK) and hybridized with the indicated random prime-labeled cDNA probes (Amersham Life Sciences). The rat *ets*-1 probe was a 1.4-kb *Bam*HI fragment of *ets*-1 cDNA cloned into the pLXSN plasmid vector. Hybridization was carried out for 1 h at 68°C in ExpressHyb hybridization solution (Clontech, Palo Alto, CA). The membranes were finally washed in a solution containing 1.7 mM NaCl, 1.7 mM sodium citrate, and 0.1% SDS at 50°C for 40 min and exposed to BioMax film (Kodak, Rochester, NY) at −80°C for 48 h. The membranes were stripped and rehybridized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, a constitutively expressed gene. The cDNA probe for GAPDH was prepared by reverse transcription-PCR as described previously (32). The primer pairs used for amplification of GAPDH were 5′-TCCACCACCCTGTTGCTGTA-3′ and 5′-ACCACAGTCCATGCCATCAC-3′. The PCR product was electrophoresed through a 1.5% agarose gel, and the GAPDH-specific band was extracted with a Qiaex II gel extraction kit (Qiagen K. K., Tokyo, Japan). The signal intensity was quantified with an imaging analyzer (Image Hyper II; DigiMo, Osaka, Japan).

SDS-PAGE and Western blotting. Confluent BAECs in 10-cm culture dishes were starved of serum for 48 h and treated with PMNs stimulated with 1 μM FMLP. The cells were washed twice with ice-cold PBS and lysed in lysis buffer [20 mM Tris·HCl (pH 7.4), 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, and 1 mM *p*-amidinophenylmethanesulfonyl hydrochloride] for 30 min on ice. The cell lysates were centrifuged at 12,000 rpm for 5 min at 4°C. After the supernatants were collected, the protein concentration was determined with a D_C protein assay kit (Bio-Rad Laboratories, Hercules, CA). Samples containing equal amounts of protein (40 μg) were separated on 10% SDS-polyacrylamide gels under reducing conditions and transferred onto Trans-Blot nitrocellulose membranes (Bio-Rad). Nonspecific binding was blocked with 0.2% Aurora blocking reagent (ICN Biomedicals, Costa Mesa, CA) in PBS containing 0.1% Tween 20 for 60 min. The membranes were incubated for 1 h with a 1:1,000 dilution of rabbit polyclonal anti-human Ets-1 (Santa Cruz Biotechnology, Santa Cruz, CA), a 1:1,000 dilution of mouse anti-human ICAM-1 (Zymed Laboratories, San Francisco, CA), or a 1:1,000 dilution of mouse anti-human E-selectin (Pharmingen, San Diego, CA) antibodies and developed with an enhanced chemiluminescence Western blotting

detection system (ECL, Amersham Pharmacia Biotech) with horseradish peroxidase (P)-conjugated second antibodies. As the second antibody, a 1:5,000 dilution of P-conjugated goat anti-rabbit IgG (Bio-Rad) for the anti-Ets-1 antibody or a 1:5,000 dilution of P-conjugated goat anti-mouse IgG (Zymed Laboratories) for anti-ICAM-1 and anti-E-selectin antibody was used. The membranes were exposed to chemiluminescence-sensitive film (Hyperfilm, Amersham) for 3–30 s. Densities of signals on the blots were measured with an image analyzer (ImageHyper II).

Statistical analysis. Results are expressed as means ± SE of n observations for each experiment. Statistical analysis was performed with the Bonferroni-Dunn procedure after ANOVA. Differences between means were considered significant at $P < 0.05$.

RESULTS

In vivo angiogenesis induced by PMNs. We previously reported (38) that PMNs stimulate in vitro angiogenesis. To determine whether PMNs induce in vivo angiogenesis, diffusion chambers containing PMNs (1×10^5 cells/ml) were implanted in the backs of rats for 7 days. Typical morphology of PMN-induced angiogenesis is shown in Fig. 1. In the surrounding tissues of control chambers containing saline, newly formed vessels were not observed (Fig. 1*A*). Implantation of the chamber containing activated PMNs induced the formation of a forestlike network of neomicrovascular vessels. Moreover, membrane hyperplasia and bleeding from the periphery of neovascular vessels were observed (Fig. 1*B*), suggesting that PMNs can stimulate angiogenesis not only in vitro but also in vivo.

Induction of Ets-1 expression by PMNs. We examined whether PMNs stimulated *ets*-1 mRNA and/or protein expression in endothelial cells. As shown in Fig. 2*A*, PMNs (1×10^5 cells/ml) induced *ets*-1 mRNA expression in BAECs and the activation of PMNs by FMLP additionally increased the *ets*-1 mRNA expression compared with PMNs alone. However, addition of FMLP to BAECs in the absence of PMNs did not affect *ets*-1 mRNA expression (Fig. 2*A*). The induction of *ets*-1 mRNA expression by activated PMNs was dependent on PMN number at 1×10^4 and 1×10^5 cells (Fig. 2*B*). To determine the time course of *ets*-1 mRNA expression, BAECs were exposed to activated PMNs for various periods (0–12 h). The induction of *ets*-1 mRNA

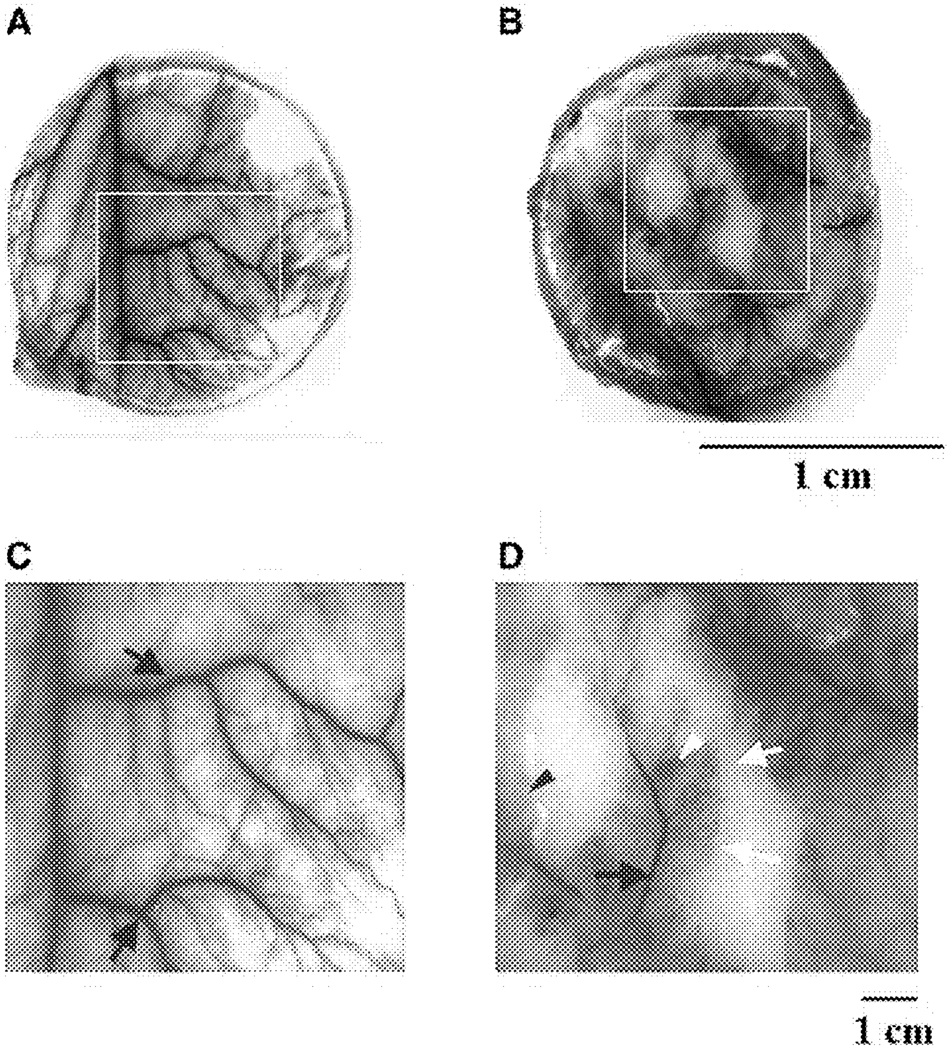

Fig. 1. Typical morphology of polymorphonuclear leukocyte (PMN)-induced angiogenesis formed on the diffusion chambers in the backs of rats. Diffusion chambers containing sterile saline as a control (*A* and *C*) and 1×10^5 PMNs (*B* and *D*) were put into the backs of rats surgically, and after 7 days the chambers were removed as described in METHODS. The framed areas of tissues in *A* and *B* are magnified in *C* and *D*, respectively. Black arrows, vessels; white arrow, neovascular tissue; black arrowhead, membrane hyperplasia; white arrowheads, bleeding.

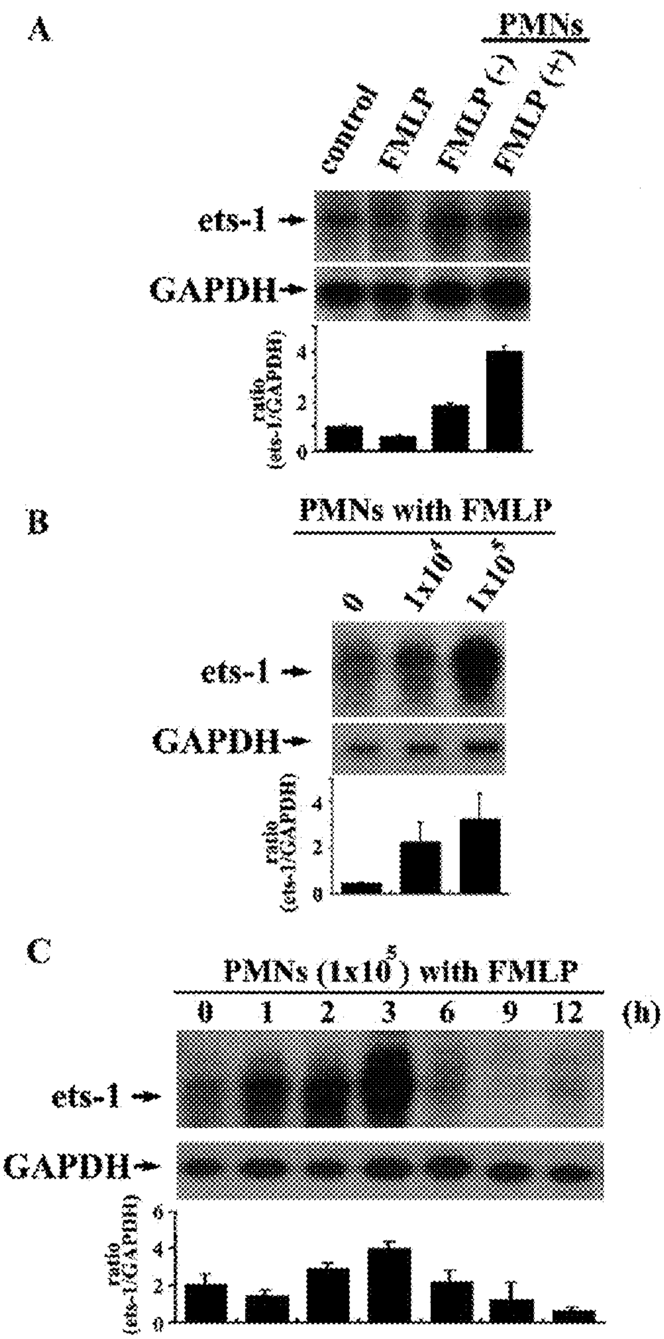

Fig. 2. Induction of *ets*-1 mRNA expression in bovine aortic endothelial cells (BAECs) stimulated by PMNs. *A*: BAECs were starved of serum for 48 h and then treated with or without 1×10^5 PMNs/ml in the presence or absence of *N*-formylmethionyl-leucyl-phenylalanine (FMLP; 1 μM) for 3 h before RNA extraction. *B*: BAECs were starved of serum for 48 h and then treated with 0, 1×10^4, or 1×10^5 PMNs/ml stimulated with 1 μM FMLP for 3 h before RNA extraction. *C*: BAECs were starved of serum for 48 h and then treated with 1×10^5 PMNs/ml stimulated with 1 μM FMLP for various periods (0–12 h) before RNA extraction. After electrophoresis of 20 μg total RNA/sample and transfer onto nylon membranes, the blots were sequentially hybridized with ^{32}P-labeled ets-1 cDNA (*top*) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (*bottom*) probes in each assay. Each column indicates the mean ± SE ratio of *ets*-1 mRNA expression to GAPDH mRNA from 2–4 independent experiments.

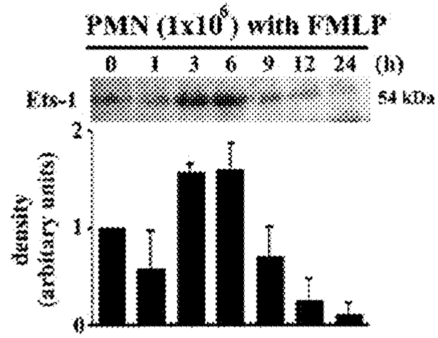

Fig. 3. Western blotting analysis of Ets-1 expression stimulated by PMNs. BAECs were starved of serum for 48 h and then treated with 1×10^5 PMNs/ml of stimulated with FMLP (1 μM) for various periods (0–24 h). Aliquots of 40 μg of protein from the BAEC lysate were fractionated by SDS-PAGE and immunoblotted with anti-Ets-1 polyclonal antibody. Each column indicates the mean ± SE density of bands in 2 independent experiments.

expression started from 1 h after addition of activated PMNs, and the peak was observed at 3 h after addition (Fig. 2*C*). To further clarify the induction of Ets-1 in BAECs stimulated by PMNs, the level of Ets-1 protein was also examined by Western blotting. The increase in Ets-1 protein was also observed at 3 and 6 h after stimulation with activated PMNs (Fig. 3).

Effects of ets-1 antisense oligonucleotide on PMN-stimulated angiogenesis and Ets-1 expression. To investigate whether *ets*-1 plays a role in PMN-induced angiogenesis, the effects of *ets*-1 antisense oligonucleotide were examined (Fig. 4). Typical morphological changes of BAECs are shown in Fig. 4, *A–C*. BAECs cultured with 0.1% FBS formed some tubelike structures (Fig. 4*A*). Addition of activated PMNs by treatment of BAECs with FMLP markedly enhanced the formation of tubelike structures with a network of branching cellular cords beneath the surface of the monolayer (Fig. 4*B*). The activated PMN-induced tube formation was inhibited by 3 μM *ets*-1 antisense oligonucleotide (Fig. 4*C*). The effects of *ets*-1 antisense oligonucleotide on activated PMN-induced angiogenesis are summarized in Fig. 4*D*. Activated PMNs stimulated angiogenesis in BAECs, and the angiogenesis was significantly blocked by *ets*-1 antisense but not by sense or mismatch oligonucleotides (Fig. 4*D*). Moreover, the activated PMN-induced *ets*-1 mRNA and Ets-1 protein expression were significantly decreased by treatment with 3 μM *ets*-1 antisense oligonucleotide but not by sense or mismatch oligonucleotides (Fig. 5, *A* and *B*).

Effects of antibodies to adhesion molecules on ets-1 mRNA expression. We previously reported (38) that FMLP treatment enhanced adhesion of PMNs to BAECs and the adhesion was inhibited by treatment with 1 μM anti-E-selectin and anti-ICAM-1 antibodies. Furthermore, we showed (38) that PMN-induced angiogenesis was strongly inhibited by anti-ICAM-1 and anti-E-selectin antibodies. To confirm the expression of ICAM-1 and E-selectin expression in endothelial cells, immunoblotting for ICAM-1 and E-selectin was per-

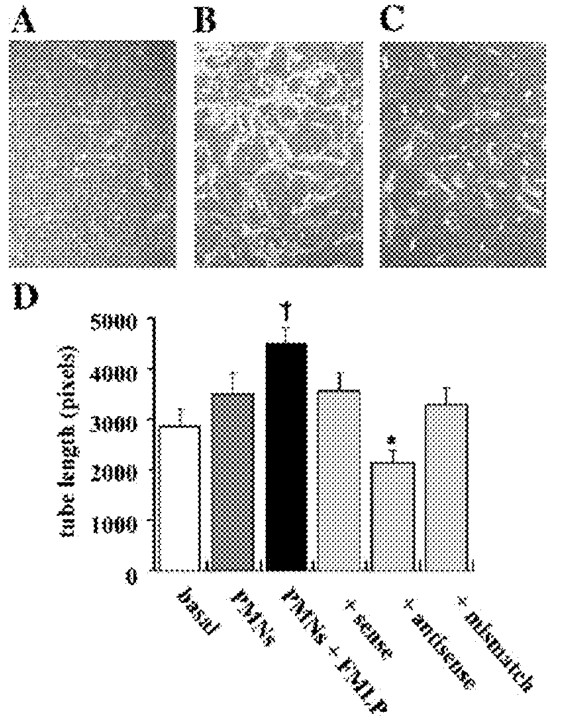

Fig. 4. Effects of *ets*-1 antisense oligonucleotide on PMN-induced angiogenesis in BAECs. Endothelial cells were cultured on collagen gel in 24-well plates to confluence, and then minimum essential medium (MEM) containing 0.1% FBS and 1×10^5 PMNs/ml stimulated with or without 1 μM FMLP were added to the cells and incubated for 72 h. *ets*-1 sense, antisense, or mismatch oligonucleotide (all at 3 μM) was added to the BAECs 6 h before addition of PMNs. The sequences of the oligonucleotides of *ets*-1 were as follows: ATG AAG GCG GCC GTC GAT CT (sense), AGA TCG ACG GCC GCC TTC AT (antisense), and ATG CAC AGC TCC GCC AGG TT (mismatch). The cultures were fixed with 0.25% glutaraldehyde and photographed (original magnification ×100). Photomicrographs show control (*A*), treatment with activated PMNs (*B*), and effects of *ets*-1 antisense oligonucleotide on activated PMN-induced angiogenesis (*C*). The tubelike structures formed were quantified by measuring the total additive length of all cellular structures including all branches with a computer-assisted image analyzer (*D*). Results are expressed as the means ± SE of 3 experiments. †P <0.05 vs. BAECs alone; *P < 0.05 vs. PMNs with FMLP.

formed (Fig. 6). Weak ICAM-1 expression was observed in BAECs under basal conditions, and the addition of activated PMNs to BAECs enhanced ICAM-1 expression from 1 to 6 h after addition (Fig. 6*A*). E-selectin expression was also enhanced by activated PMNs from 18 h after addition (Fig. 6*B*).

We next examined the effects of antibodies to adhesion molecules on *ets*-1 mRNA expression in BAECs treated with FMLP-stimulated PMNs. Anti-ICAM-1 antibody inhibited the *ets*-1 mRNA expression induced by activated-PMNs. On the other hand, anti-E-selectin antibody did not reduce the activated PMN-induced *ets*-1 mRNA expression (Fig. 7).

Effects of antibodies to adhesion molecules on H_2O_2-induced angiogenesis. We previously reported (37) that addition of H_2O_2 to BAECs enhanced angiogenesis. To determine the roles of ICAM-1 and E-selectin in the induction of angiogenesis by stimulation of endothelial cells without PMNs, the effects of anti-ICAM-1 and anti-E-selectin antibodies on H_2O_2-induced angiogenesis were examined (Fig. 8). H_2O_2-induced angiogenesis was inhibited in a concentration-dependent manner by treatment with anti-E-selectin antibody but not by anti-ICAM-1 antibody (Fig. 8, *A* and *B*). Moreover, the expression of *ets*-1 mRNA induced by H_2O_2 was not inhibited by either antibody (Fig. 9).

Effects of superoxide dismutase or catalase on ets-1 mRNA expression in BAECs stimulated with PMNs. To investigate the role of H_2O_2 released from PMNs in stimulation of Ets-1 expression, the effects of catalase and superoxide dismutase (SOD) on *ets*-1 mRNA expression stimulated by PMN were studied. Activated

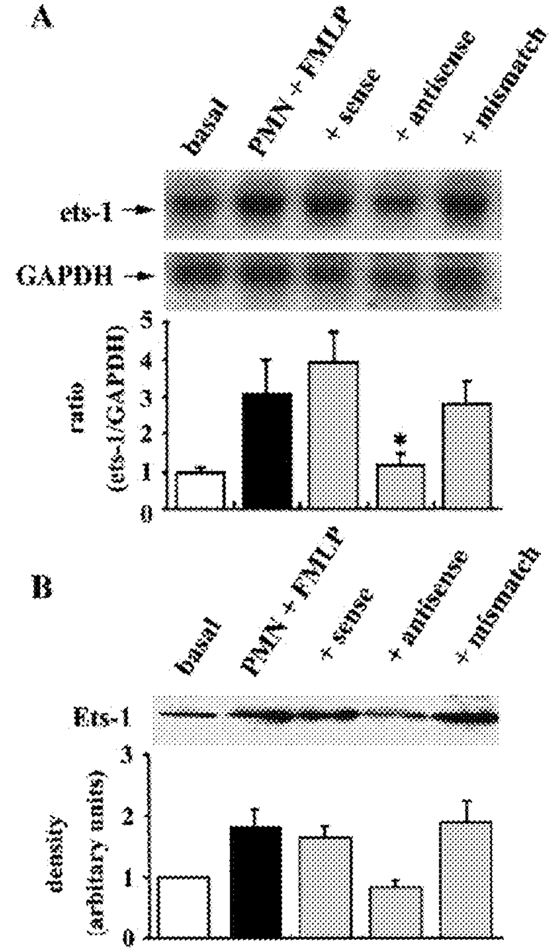

Fig. 5. Effects of *ets*-1 antisense oligonucleotide on the induction of Ets-1 expression in BAECs stimulated with PMNs. BAECs were starved of serum for 48 h and pretreated with *ets*-1 sense, antisense, or mismatch oligonucleotide (all at 3 μM) for 6 h. The BAECs were then treated with 1×10^5 PMNs/ml stimulated with 1 μM FMLP for 3 h before total RNA and protein extraction. The sequences of the sense, antisense, and mismatch oligonucleotides of *ets*-1 are shown in Fig. 4. *A*: Northern blotting analysis of *ets*-1 mRNA expression in BAECs. Each column indicates the mean ± SE ratio of *ets*-1 mRNA expression to GAPDH mRNA from 4 independent experiments. *P < 0.05 *vs.* PMN with FMLP. *B*: Western blotting analysis of Ets-1 protein expression in BAECs. Each column indicates the mean ± SE density of bands in 2 independent experiments.

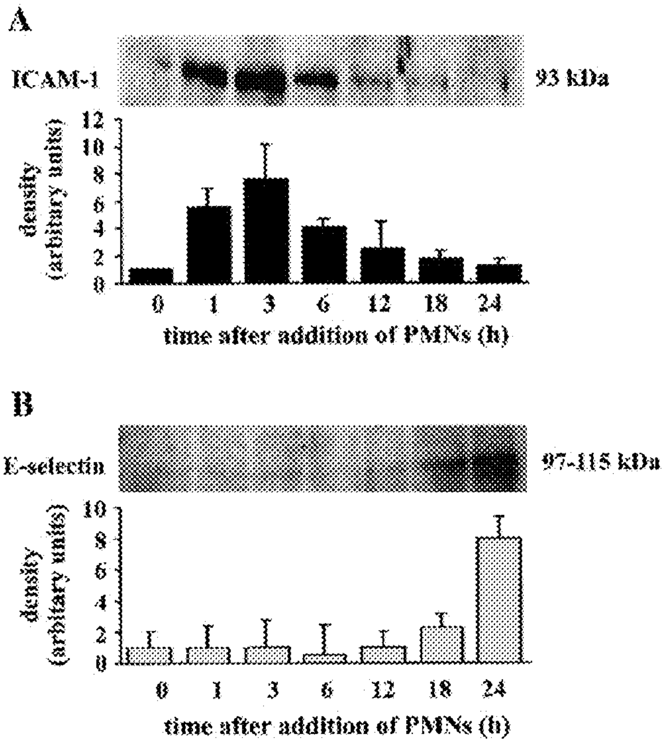

Fig. 6. Expression of ICAM-1 and E-selectin in BAECs. BAECs were starved of serum for 48 h, and then FMLP (1 μM)-stimulated PMNs were added for various periods (0–24 h). The obtained proteins (40 μg) were fractionated by SDS-PAGE and then immunoblotted with anti-ICAM-1 monoclonal antibody (*A*) or anti-E-selectin monoclonal antibody (*B*). Each column indicates the mean ± SE density of bands in 2 independent experiments.

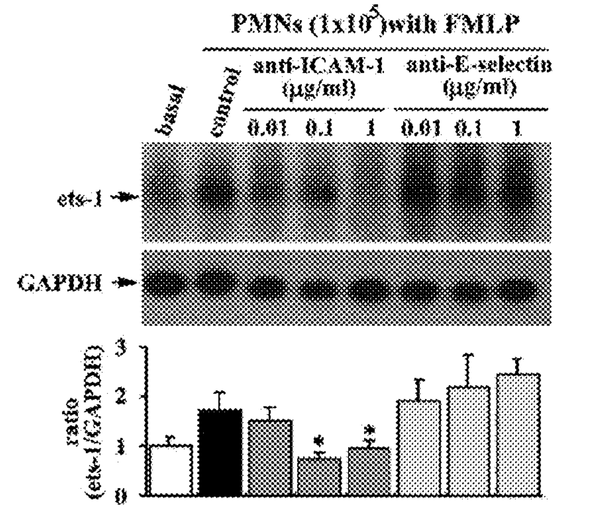

Fig. 7. Effects of antibodies to adhesion molecules on PMN-induced *ets*-1 mRNA expression in BAECs. BAECs were starved of serum for 48 h and pretreated with 0.01–1 μg/ml anti-E-selectin or anti-ICAM-1 antibody. Subsequently, the BAECs were stimulated with 1×10^5 PMNs/ml stimulated with 1 μM FMLP for 3 h before RNA extraction. After electrophoresis of 20 μg RNA/sample and transfer onto nylon membranes, the blots were sequentially hybridized with ^{32}P-labeled *ets*-1 cDNA (*top*) and GAPDH cDNA (*bottom*) probes. Each column indicates the mean ± SE ratio of *ets*-1 mRNA expression to GAPDH mRNA from 4 independent experiments. $^*P < 0.05$ vs. control.

PMN-induced *ets*-1 mRNA expression was inhibited by catalase but not by SOD (Fig. 10).

DISCUSSION

Our previous study (38) showed that PMNs stimulate angiogenesis in BAECs. However, the mechanisms underlying induction of PMN-induced angiogenesis remained unclear. The initiation of angiogenesis requires digestion of the extracellular matrix via induction of protease activities for endothelial cell migration into the interstitial space (4). Recently, the transcription factor Ets-1, which regulates the gene expression of proteases such as u-PA, MMP-1, MMP-3, and MMP-9, was shown to mediate angiogenesis induced by VEGF and epidermal growth factor (EGF) (14, 27, 34). In the present study, we found that Ets-1 expression in endothelial cells was stimulated by activated PMNs and both PMN-induced angiogenesis and Ets-1 expression were strongly reduced by *ets*-1 antisense oligonucleotide. Thus Ets-1 also seems to play a central role in PMN-induced angiogenesis in addition to angiogenic growth factor-induced angiogenesis.

PMNs adhere to endothelial cells via adhesion molecules such as ICAM-1 and E-selectin. Adhesion molecules were initially thought to function only in cell adhesion between vascular endothelial cells and leukocytes (3, 6, 16). However, adhesion of PMNs to endothelial cells was reported recently to trigger various physiological changes including an increase in intracellular Ca^{2+} concentration and activation of transcription factor nuclear factor-κB (1, 7, 22, 25, 28). Our previous study (38) showed that anti-ICAM-1 and anti-E-selectin antibodies, which inhibited adhesion between PMNs, prevented PMN-induced angiogenesis by endothelial cells. In fact, the expression of ICAM-1 and E-selectin was confirmed on BAECs stimulated by PMNs. Thus both ICAM-1

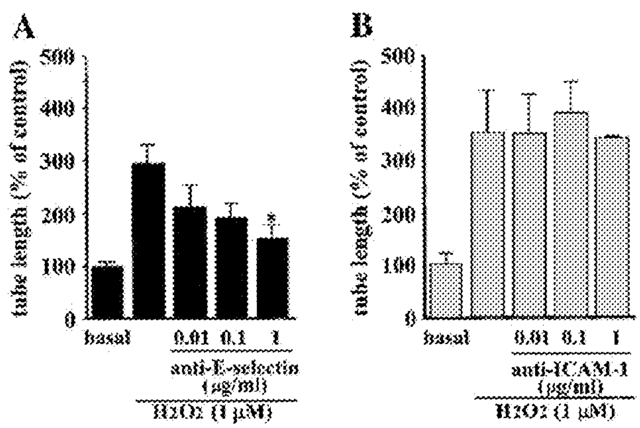

Fig. 8. Effects of antibodies to adhesion molecules on angiogenesis induced by H_2O_2. BAECs were preincubated with or without anti-ICAM-1 (0.01–1 μg/ml; *A*) or anti-E-selectin (0.01–1 μg/ml; *B*) monoclonal antibodies for 30 min. After incubation, H_2O_2 (1 μM) was added to the cultures and incubated for 3 days. Results are expressed as means ± SE of 3 experiments. $^*P < 0.05$ vs. H_2O_2-stimulated BAEC without antibodies.

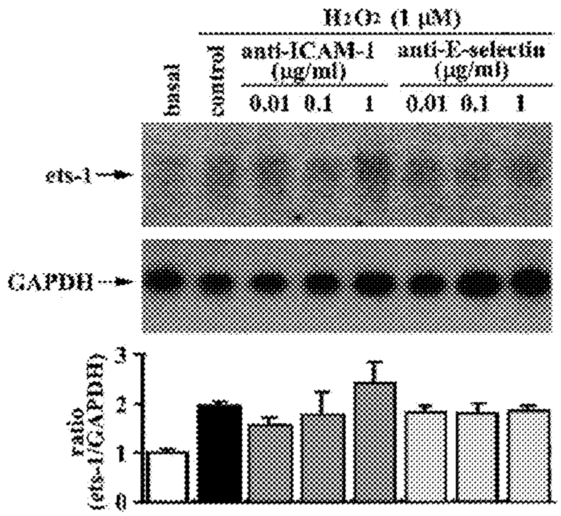

Fig. 9. Effects of antibodies to adhesion molecules on H_2O_2-induced *ets*-1 mRNA expression in BAECs. BAECs were starved of serum for 48 h and pretreated with 0.01–1 μg/ml anti-E-selectin or anti-ICAM-1 antibody. Subsequently, the BAECs were stimulated with 1 μM H_2O_2 for 3 h before RNA extraction. After electrophoresis of 20 μg RNA/sample and transfer onto nylon membranes, the blots were sequentially hybridized with ^{32}P-labeled *ets*-1 cDNA (*top*) and GAPDH cDNA (*bottom*) probes. Each column indicates the mean ± SE ratio of *ets*-1 mRNA expression to GAPDH mRNA from 4 independent experiments.

and E-selectin seem to be essential factors for PMN-induced angiogenesis. Importantly, the activated PMN-induced increase in *ets*-1 mRNA expression was inhibited by anti-ICAM-1 antibody but not by anti-E-selectin antibody. ICAM-1 but not E-selectin might act as a signaling receptor for the induction of Ets-1. We previously reported (37) that H_2O_2 stimulates angiogenesis through the induction of Ets-1. Interestingly, H_2O_2-induced angiogenesis was inhibited by anti-E-selectin antibody but not by anti-ICAM-1 antibody. Nguyen et al. (26) previously reported that formation of tubelike structures by BAEC cultured on fibronectin-coated plates was inhibited by antibodies to sialyl Lewis$^{X/A}$ and E-selectin. E-selectin seems to function in capillary morphogenesis via endothelial cell-cell interaction during angiogenesis. These findings indicate that although ICAM-1 and E-selectin are essential factors, they have a different roles in PMN-induced angiogenesis, i.e., ICAM-1 might act as a signaling receptor for induction of Ets-1 expression, and E-selectin might act in formation of tubelike structures via endothelial cell-cell adhesion.

The activated PMN-induced *ets*-1 mRNA expression was further stimulated by treatment with anti-E-selectin antibody. There are several possible mechanisms that could account for these observations. First, the signal from E-selectin by cell-cell adhesion between endothelial cells during formation of tubelike structures may negatively regulate *ets*-1 mRNA expression induced by activated PMNs. However, this possibility was excluded by the lack of stimulatory effect of anti-E-selectin antibody on H_2O_2-induced *ets*-1 mRNA expression, although H_2O_2 induces the formation of tubelike structures. Second, the signal from E-selectin by the interaction between PMN and endothelial cells may negatively regulate *ets*-1 mRNA expression induced by activated PMNs. In fact, H_2O_2-induced *ets*-1 mRNA expression was not affected by treatment with E-selectin antibody. Thus future studies are needed to determine the role of E-selectin in PMN-induced *ets*-1 mRNA expression.

Activated PMNs have been shown to release reactive oxygen species (ROS) including H_2O_2 (11, 21, 23). Our previous studies indicated that H_2O_2 (0.1–10 μM) stimulates angiogenesis via induction of Ets-1 (37) and that PMN-stimulated angiogenesis was inhibited by catalase but not by SOD (38). PMN-induced *ets*-1 mRNA expression was also inhibited by catalase. Thus H_2O_2 released from PMNs seems to be involved in the stimulation of angiogenesis through the induction of Ets-1 expression. In the present study, we used non-stimulated endothelial cells to investigate the mechanisms underlying activated PMN-induced angiogenesis, although the activation of endothelial cells is also necessary for the interaction with PMNs. Importantly, H_2O_2 has been shown to stimulate the expression of adhesion molecules including ICAM-1 (23, 29). In fact, leukocyte accumulation under inflammatory conditions seems to be mediated by ROS such as H_2O_2 and superoxide (20, 31). The increase of ICAM-1 protein level was observed ~2 h before stimulation of Ets-1 protein level by treatment with activated PMNs. It is possible that PMN-induced Ets-1 expression is mediated by stimulation of ICAM-1 expression induced by H_2O_2 released from PMNs. Future studies are needed to determine the role of H_2O_2 in the regulation of adhesion molecule expression during PMN-induced angiogenesis.

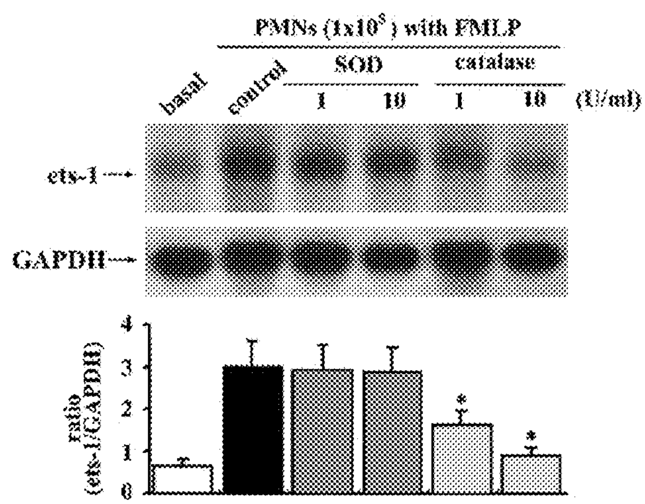

Fig. 10. Effects of superoxide dismutase (SOD) and catalase on *ets*-1 mRNA expression in BAECs stimulated with PMNs. BAECs were serum-starved for 48 h, and 1 or 10 U/ml SOD or catalase was added. Subsequently, the BAECs were stimulated with 1 × 10^5 PMNs/ml with 1 μM FMLP for 3 h before RNA extraction. Each column indicates the mean ± SE ratio of *ets*-1 mRNA expression to GAPDH mRNA from 3 independent experiments. $^*P < 0.05$ vs. control.

In conclusion, our findings suggest that *ets*-1, ICAM-1, and E-selectin have critical roles in PMN-induced angiogenesis. ICAM-1 may act as a signaling receptor to induce Ets-1 induction, whereas E-selectin seems to be involved in the formation of tubelike structures via cell-cell interactions between endothelial cells.

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EXHIBIT 4

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ANTI-ANGIOGENIC EFFECTS OF SOME ANTI-MICROBIAL DRUGS.

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It is known that antimicrobial drugs are able to modify immunological system directly or indirectly. The aim of our study was to determine the influence of some antibiotics on new blood vessels formation. We checked the influence of some antibiotics in therapeutical doses (Clindamycin, Cefuroxime, Rifampicin, Rifamycin, Doxycycline, Cefoperazone) on angiogenic activity of human blood mononuclears (MNC) in LIA (leukocyte induced angiogenesis) test and tumour cells isolated from human lung carcinomas (adenocarcinoma and ca planoepitheliale) in TIA (tumour induced angiogenesis) test in Balb/c mice (according to Sidky and Auerbach).

As far as TIA concerned all examined antibiotics (except for Rifampicine) inhibited angiogenic activity of human tumour cells and the effect was highly statistically significant.

We also showed that most of previously mentioned antibiotics as well as cefradine and pyrazynamide, caused statistically significant inhibitory effect on angiogenic activity of human MNC. Additional experiments performed on partly purified cell populations, revealed different suppressory mechanisms.

In case of Rifampicin, suppression was connected with the presence of CD4+ lymphocytes. As far as pyrazynamide is concerned, suppressory effect was dependent on the presence of monocytes, and in case of cefradine both monocytes and CD8+ lymphocytes were responsible.

Further studies will show the potential therapeutic significance of some antibiotics in angiogenesis - dependent diseases.